Stem Canker Diseases of Eucalypts in Tasmania

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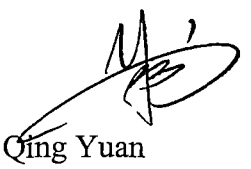
DECLARATION

This thesis contains no material which has been accepted for the award of any degree or diploma in any university and, to the best of my knowledge, contains no copy or paraphrase of material previously published or written by another person, except where due reference is made in the text of the thesis.



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June 1998

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ABSTRACT

In order to evaluate the range of stem canker fungi in natural eucalypt forest and plantations in Tasmania, a systematic survey was conducted. A total of 210 samples representing 30 fungal species were collected. The three species most frequently encountered were *Endothia gyrosa*, *Cytospora eucalypticola* and *Valsa ceratosperma*. Ten of the fungal species detailed in this survey were newly published and five were reported for the first time in Australia.

Pathogenicity studies were conducted with 11 fungal species collected from the survey. Three species (*E. gyrosa*, *Phoma* sp. and *Seiridium eucalypti*) could cause significant cankers on both *E. nitens* and *E. globulus*. Influences by host species, provenance, age, vigour and bark type on canker development are discussed.

The incidence of canker is higher in rough-barked *E. nitens* compared to smooth-barked trees. Longitudinal cracking in rough bark provides natural infection courts. However, once infected artificially, smooth-barked *E. nitens* is more susceptible than rough-barked. This susceptibility is attributed to the anatomical structure of smooth bark facilitating post-infection penetration.

A high incidence of severe *E. gyrosa* cankers was observed in 1993 at Tewkesbury (northwestern Tasmania) within a vigorously growing plantation of mixed (smooth or rough barked) provenances of 16 yr old *E. nitens*. This observation initiated an in-depth investigation of *E. gyrosa*.

Stem inoculations with isolates of *E. gyrosa* originating from different locations across Australia showed that all can infect *E. nitens* and *E. globulus*. However, isolates from Tasmania, Victoria and Western Australia were generally more aggressive than those from the Australian Capital Territory and New South Wales.

Endothia gyrosa isolates from Australia and overseas were compared. Four main types of colony morphology were recognised among 133 isolates based on the colour and density of the vegetative mycelium.

Vegetative incompatibility was detected using a pH amended medium. Sixteen isolates from different origins in Australia, South Africa, North America and Europe were grouped into 9 vegetative compatibility groups with this method.

There was correspondence between the grouping of these sixteen isolates as determined by colony morphology and vegetative compatibility and those revealed by DNA polymorphisms in RFLP and RAPD analyses. Overseas and Australian isolates appear closely related. Within Australia isolates from as geographically distant locations as Western Australia and Victoria were grouped together. The significance of observed levels of intraspecific variation in *E. gyrosa* is discussed.

The potential threat of canker fungi, especially *E. gyrosa*, to the plantation forestry is reviewed.

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List of publications from this thesis

Refereed papers

1. Yuan, Z.Q. & Mohammed, C. 1997*a*. Investigation of fungi associated with stem cankers of eucalypts in Tasmania, Australia. *Australiasian Plant Pathology* **26**(2): 78-84.

2. Yuan, Z.Q. & Mohammed, C. 1997*b*. *Wuestneia epispora* sp. nov. on stems of eucalypts from Australia. *Mycological Research* **101**(2): 195-200.

3. Yuan, Z.Q. & Mohammed, C. 1997*c*. *Seiridium papillatum*, a new species (mitosporic fungus) described on stems of eucalypts in Australia. *Australian Systematic Botany* **10**(1): 69-75

4. Yuan, Z.Q. & Mohammed, C. 1997*d*. New species and new records of ascomycetes on stems of eucalypts from Tasmania, Australia. *Mycotaxon* **63**: 9-23.

5. Yuan, Z.Q. & Mohammed, C. 1997*e*. *Therrya eucalypti* sp. nov. on stems of eucalypts from Tasmania, Australia. *Mycotaxon* **64**: 173-177.

6. Yuan, Z.Q. & Mohammed, C. 1997*f*. Two new mitosporic coelomycetes with appendages on stems of eucalypts from Australia. *Mycological Research* **101**(12): 1531-1534.

7. Yuan, Z. Q. & Mohammed, C. 1998. Ability of ascospores and conidia of *Endothia gyrosa* to infect wounds in young *Eucalyptus nitens*. *New Zealand Journal of Forestry Science* (In press).

Conference paper (abstract)

1. Yuan, Z. Q. & Mohammed, C. 1997*g*. Pathogenicity of fungi associated with stem cankers of eucalypts in Tasmania. *In* The 11th Biennial conference proceedings of Australasian Plant Pathology Society, 29 Sept.-2 Oct. 1997, Perth, Western Australia, p. 25.

GENERAL INTRODUCTION

What is a canker?

Canker diseases in trees have been recognised for centuries. More than 300 years ago (1629), a British gardener, John Parkinson wrote, *"The canker is a shrewd disease when it happeneth to tree. It must be looked into before it hath runne too farre".*

Cankers on trees are the visible manifestation of necrotic periderm, cortex, phloem, and vascular cambium tissue (Boyce 1961; Biggs 1992). Cankers can cause reduced tree growth rates, tree mortality, and may destroy forests if infection is widespread.

In most shade tree or forest pathology textbooks, cankers are classified according to types or classes (Manion 1991, Tainter and Baker 1996):

1. saprobic cankers,
2. annual cankers,
3. perennial cankers.

If cankers are perennial they are diffuse or target shaped.

Saprobic cankers often occur on lower branches or in the upper crowns of trees. The fungi causing saprobic cankers are associated with tree decline. They cannot penetrate the morphological defense barriers of healthy trees, nor can they tolerate the chemical defense mechanisms of normal healthy trees.

Annual cankers are usually superficial. Fungal infections, often resulting from the opportunist infection of wounds, are contained by callus development of host tissue after the first year's invasion.

Perennial cankers are more destructive and conspicuous. Fungi continue to infect new tissue year after year at the margin of the canker.

Diffuse cankers destroy the cambium of stems as lesions develop along the stems. A canker fungi grows so quickly that the host forms little or no callus tissue. When the lesions girdle the main stem, the tree will die.

Target shaped cankers are formed when the host responds by forming callus tissue, and then the fungus overcomes the resistant reponse and overgrows the callus tissue. When this see-saw battle occurs over many years, annual layers of callus tissue are formed, often concentric in shape, giving the appearance of a target from which it is named.

Cankers may be incited by fungi from the Ascomycetes, Deuteromycetes, Basidiomycetes, and less frequently, Oomycetes. Generally, stem cankers are caused by Ascomycetes, Deuteromycetes, and Oomycetes, whereas canker rots are caused by Basidiomycetes.

Most fungi that cause stem cankers are restricted to bark and xylem tissues that succumb due to the effects of toxins or secreted enzymes. Such canker pathogens are considered to be necrotrophic or facultative parasites (Manion 1991). They include many extensively studied pathogens [eg. *Nectria galligena* Bres., *Cryphonectria parasitica* (Murrill) Barr, *Leucostoma persoonii* Höhn.]. They are adapted for harmless saprobic existence on the dead tissue of trees, but are also able to attack the living cells of trees to cause canker. This is particularly the case when trees are weakened due to other stress factors such as insect attack and drought. Weakened trees are unable to produce sufficient morphological and chemical barriers to prevent invasion of canker fungi (Manion 1991).

Stem canker fungi colonise the plant via open wounds, dead branches, branch stubs, twigs, leaf scars or, less commonly, through leaves. Direct penetration of intact bark or epidermis has not been demonstrated for any of the facultative parasites that cause stem cankers. *Nectria galligena* is able to enter the apple host through wounds inflicted by pruning, frost injury, breakage caused by ice and snow (Lortie 1964), and small dead branch stubs (Grant and Childs 1940, Zalasky 1968). *Hypoxylon*

mammatum (Wahlenberg) J.H. Miller enters aspen trees through wounds caused by insects, axe cuts, snow and ice breakage, and dead branches (Bier 1940). *Cryphonectria parasitica* can become established through any type of wound that is deeper than the outer green cortex (Biggs 1992).

Damaging tree canker pathogens

A classic example of a devastating tree canker disease is chestnut blight, caused by *Cryphonectria parasitica*. This disease was introduced to North America and Europe from eastern Asia. Within 50 years of its discovery in New York in 1904, chestnut blight had ravaged 3.6 million hectares of the American chestnut (*Castanea dentata* Borkh.) forests in eastern North America. In 1912, the total losses of timber alone for three states of the USA was valued at US$82.5 million (Anagnostakis 1987).

The disease was introduced into southern Europe in about 1938 and spread at a similar rate to that already seen in North America. It threatened the European chestnut (*C. sativa* Mill) with extinction (Heiniger and Rigling 1994). Prior to the introduction of the disease, there were 45,000 ha. of grafted orchard chestnut trees and 400,000 ha. of chestnut coppice in Italy. By 1950, 121,000 ha. had been killed (Gravatt 1952).

The pathogen was probably introduced into North America with horticultural material from China and Japan on several occasions (Anagnostakis 1987; Milgroom *et al.* 1992). It was sometimes destructive in China on Chinese chestnut (*C. mollissima* Bl.) and in Japan on Japanese chestnut (*C. crenata* Sieb. et Zucc.) (Uchida 1977). This is a typical example of a disease epidemic occurring when a virulent pathogen is introduced to the habitat of a susceptible host.

Cryphonectria cubensis (Bruner) Hodges (syn. *Diaporthe cubensis* Bruner), another member of the genus has been the most damaging canker pathogen of eucalypt plantations in many parts of the world including Brazil, Cameroon, Costa Rica, Cuba, India, Indonesia, Puerto Rico, South Africa, Surinam, Thailand and the United States of America (Boerboom and Maas 1971; Hodges *et al.* 1976, 1979; Sharma *et al.* 1985;

Wingfield *et al.* 1989; Conradie *et al.* 1990). *Cryphonectria cubensis* was first proposed as *D. cubensis* when it was found causing canker disease of *Eucalyptus* in Cuba as early as 1917 (Bruner 1917). However, the pathogen was not recognised as a major threat to eucalypt plantations until the early 1970s in Brazil (Hodges *et al.* 1976). At that time (1974) Brazil had planted about 1,052,000 ha. of eucalypts, (the largest area of eucalypt plantations in the world, FAO 1981). *Cryphonectria cubensis* greatly influenced the selection of eucalypt species and their management (Krugner 1991). The use of resistant or less susceptible eucalypt species and the clonal propagation of resistant genotypes is the only means of reducing losses from the disease (Hodges *et al.* 1976; Alfenas *et al.* 1997).

Canker fungi reported on *Eucalyptus*

Table 1 shows that worldwide, a large number of pathogenic fungal species are associated with stem canker diseases on a range of eucalypt species. Several of the important canker diseases occurring in different parts of the world have been discussed in a FAO/IPGRI (International Plant Genetic Resources Institute) technical guideline for the safe movement of germplasm on eucalypt diseases (Ciesla *et al* 1996). They are *Botryosphaeria* canker, *Cryphonectria* canker, *Endothia* canker, *Coniothyrium* canker, *Seiridium* canker and pink disease.

Relatively few of these pathogens have been reported from Australia: *Endothia gyrosa* (Schw.: Fr.) Fr., *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not., *Cytospora eucalypticola* van der Westhuizen, *Hypoxylon howeianum* Peck, *Ramularia pitereka* J. Walker & Bertus and *Seiridium eucalypti* Nag Raj. This is unexpected as the genus *Eucalyptus* L'Her. is endemic to Australia and about 90 percent of the Australian forests consists of more than 600 eucalypt species (Pryor 1976; FAO 1981).

Indeed cankers (and their causal organisms) were not given significant attention in Australia until the 1980s (Davison 1982; Davison and Tay 1983; Walker *et al.* 1985; Fraser and Davison 1986; Old *et al.* 1986, 1990), although there were a few earlier reports concerning canker fungi on eucalypts (Browne 1968; Smith 1970). In

Tasmania, fungi associated with plant diseases recorded prior to 1978 have been annotated and listed by Sampson and Walker (1982). In this list, only one fungus, *Melanconium eucalypti* Mass. & Rodw. was recorded on *Eucalyptus urnigera* Hook. f., possibly from stems.

Incidence and pathogenicity of canker fungi on *Eucalyptus* in Australia

Today cankers associated with *B. dothidea*, *C. eucalyticola* and *E. gyrosa* are recognised as commonly occurring on the stems and crowns of eucalypts in natural forests in many parts of Australia. Such cankers are considered to be one of the contributing factors to eucalypt dieback in rural areas (Old 1998, pers. comm.).

Botryosphaeria dothidea [referred to in early records as *B. ribis* (Tode : Fr.) Gross & Dugg. in Australia] has also been commonly isolated from cankered stems of various eucalypts in Western Australia and southeastern Australia (Davison and Tay 1983; Old 1986 unpublished data; Yuan 1989). It caused the girdling and death of *E. radiata* Sieb. ex DC in Western Australia (Shearer *et al.* 1987).

Cytospora eucalypticola has been reported as damaging to eucalypts in Africa (van der Westhuizen 1965a,b) whereas, in Australia, it is considered to be a weak or non-aggressive pathogen to eucalypts (Old *et al.* 1986).

Seiridium eucalypti was first collected on stems of *E. delegatensis* R. Baker from natural forests in northern Tasmania in 1987 (Yuan and Old 1995). Subsequently, in an artificial inoculation study using 13-month-old seedlings of five eucalypt species, *S. eucalypti* was demonstrated to be the most pathogenic canker fungi of five tested fungi including *E. gyrosa*, *B. dothidea* and *C. eucalypticola* (Yuan and Old 1995).

Although *Endothia gyrosa* is a fungal species widely associated with cankers on natural trees in south eastern and western Australia (Davison 1982; Davison and Tay 1983; Old *et al.* 1986) it is not been considered as an aggressive pathogen of eucalypts in Australia (Old *et al.* 1990). However in 1993 this fungus was found to cause

significant levels of stem damage and death of vigorously growing trees in Tasmania (Wardlaw 1998).

The potential significance of canker diseases to plantation forestry in Australia

Both on an international and domestic level, Australia has a strong commitment to the role of forestry in sustainable development. In order to remain competitive, this has meant a move towards more intensive silvicultural management of regrowth and plantation eucalypts. The generally small size of trees and high cost inputs involved in plantations means that the economic tolerance to loss resulting from pests and diseases is low. The population structure of natural and traditionally managed forests has not favoured the devastating disease epidemics which often occur in simple plantation forests (*sensu* Kanowski 1995) of monocultures (Zadoks and Schein 1979; Manion 1991).

Compared with other timber producing countries, Australia has established a significant hardwood plantation resource (with an estimated 190,000 ha. established by 1997).

Tasmania is one of the two principal hardwood plantation centres (along with Western Australia) with about 68,000 ha. of plantation eucalypts in 1997 and *ca.* 8000 ha. being planted annually (Burns *et al.* 1997; Stafford and Neilson 1994).

North Forest Products (NFP), the largest private landowner in Tasmania (with 50,000 ha.) is developing tree farms with selected seedlings of *Eucalyptus globulus* Labill. and *E. nitens* (Deane & Maiden) Maiden for the production of fine printing paper. These tree farms are being planted at a rate of more than 6 million trees a year which represents an investment of more than $8 million annually.

Similarly, Australian Paper, another private company, is now planting eucalypt trees at the rate of 1,300 hectares/annum, or an average of 1.4 million trees each year in Tasmania.

Forestry Tasmania, a government owned business enterprise, has established 12,036 ha. of eucalypt plantations (Taylor 1998, pers. comm.). Under an 'Intensive Forest Management Program', Forestry Tasmania commenced the establishment of eucalypt plantations for production of sawlogs and veneer in 1991. They planted approximately 7000 ha. of eucalypts, mainly *E. nitens* and a small area of *E. globulus* in 1996 (Farmer and Smith 1997). About 20,000 ha. of new plantations are to be planted between 1997-2002. *Eucalyptus globulus* plantations will be included in the new round of plantings for solid wood products (Wardlaw 1998, pers. comm.).

RESEARCH AIMS

In Tasmania, priority is being given to develop a cost-effective means of conducting health surveys in eucalypt plantations. One potentially significant constraint to effective health surveys in eucalypt plantations is the lack of knowledge of the identity and status of fungi, especially canker fungi, associated with damage to eucalypts. The objectives of this thesis are therefore:

1. To survey, collect and identify fungi associated with stem and branch cankers of eucalypts in Tasmania;
2. To test the pathogenicity of selected canker fungi in order to understand their potential impact on fast growing plantation eucalypt species;
3. To carry out an in-depth investigation of the species *Endothia gyrosa* and assess its potential as a threat to Australian forestry.

Table 1: List of fungi associated with canker and die-back of stems and branches (including shoots and twigs) of *Eucalyptus* spp in the world

Fungal species	Disease / Host	Distribution	References
Coniella australiensis	Dead branches of *E. deglupta* following insect damage	Papua New Guinea	Shaw, 1984
Bagnisiopsis eucalypti	Twigs of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Botryodiplodia theobromae	Stem canker of *E. grandis*& *E. tereticornis*	India	Sharma *et al.*, 1989
Botryosphaeria dothidea	Basal canker, twig dieback of *Eucalyptus* spp.	USA	Farr *et al.*, 1989
	Canker of *E. grandis*	USA	Barnard *et al.*, 1987
	Stem canker of *E. marginata*	Australia	Davison & Tay, 1983
	E. radiata	Australia	Shearer *et al.*, 1987
	Stem cankers of *E. grandis*, *E. macarthurii*, *E. nitens*, *E. camaldulenis* (hybrids) & *E. urophylla* (hybrids)	South Africa	Smith *et al.*, 1994
	Stem canker of *E. camaldulensis*	USA	Webb, 1983
Botryosphaeria rhodina (syn. *Physalospora rhodina*)	Stem canker of *E. globulus*, *E. saligna* & *E. punctata*	Kenya, Nigeria, USA	Gibson, 1975
Botryosphaeria ribis var. *chromogena*	Dieback of *Eucalyptus* sp.	USA	Spaulding, 1961
Botrytis cinerea	On twigs of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
	Blight & decay of *E. alba, E. botryoides, E. camaldulensis, E. citriodora, E. globulus & E. tereticornis*	Argentina	Spaulding, 1961
	E. globulus	Kenya	Spaulding, 1961
Coniothyrium sp.	Stem canker of *E. grandis* & *E. smithii*	South Africa	Brits & Grey, 1992

Table 1: continued

Fungal species	Disease / Host	Distribution	References
Cryphonectria cubensis	Basal & stem canker of *Eucalyptus* spp.	USA	Farr *et al.*, 1989
	Stem canker of *E. citriodora*, *E. deglupta, E. grandis* & *Eucalyptus* spp.	India	Sharma *et al.*, 1989
	Stem canker of *E. grandis*, *E. saligna* & *E. urophylla*	South Africa	Brits & Grey, 1992
Cryphonectria gyrosa	Stem canker of *E. alba*, *E. deglupta, E. grandis, E. tereticornis* & *E. torelliana*	India	Sharma *et al.*, 1989
	Stem canker of *E. camaldulensis*	Vietnam	Old & Yuan, 1994
	Coppice shoot death of *E. grandis*	USA	Barnard *et al.*, 1987
Cylindrocladium scoparium	Stem lesions of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
	Eucalyptus sp.	Argentina	Spaulding, 1961
	Eucalyptus sp.	Brazil	Spaulding, 1961
	Eucalyptus citriodora, E. coccifera, E. gigantea, E. globulus, E. gunnii, E. longifolia, E. obliqua, E. pauciflora, E. regnans, E. resinifera, E. robusta, E. saligna, E. tereticornis & *E. viminalis*	Japan	Spaulding, 1961
Dothiorella eucalypti	Twig dieback of *E. camaldulensis*	USA	Farr *et al.*, 1989

Table 1: continued

Fungal species	Disease / Host	Distribution	References
Endothia gyrosa (anamorph *Endothiella gyrosa*)	Stem canker of *E. diversicolor*	Portugal	Spaulding, 1961
	Stem canker of *E. grandis*	South Africa	van der Westhuizen *et al.* 1993
	Stem canker of *E. dunnii, E. elata, E. fastigata, E. grandis, E. macarthurii, E. saligna & E. smithii*	South Africa	Brits & Grey, 1992
	Stem canker of *E. delegatensis E. maculata, E. obliqua, E. pauciflora, E. regnans, E. rossii, E. saligna & E. viminalis*	Australia	Old *et al.* 1986
	Stem canker of *E. nitens*	Australia	Wardlaw, 1998
Endothia sp.	Canker of *E. grandis*	USA	Farr *et al.*, 1989
Fusicoccum sp.	Stem canker of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Harknessia eucalypti	Twigs of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Hendersonia sp.	Twig dieback of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Hypoxylon howeianum	Stem canker of *E. regnans*	Australia	Browne, 1968
Hypoxylon mediterraneum	Stem canker of *E. camaldulensis, E. gomphocephala, & E. robusta*	Morocco	Spaulding, 1961
Hypoxylon sertatum	Dieback of *E. robusta*	Morocco	Spaulding, 1961
Hysterium angustratum	Stem canker of *Eucalyptus* spp.	India	ICFRE, 1990
Macrophoma molleriana	Twig dieback of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Macrovalsaria megalospora	Stem canker of *Eucalyptus* sp.	India	ICFRE, 1990

Table 1: continued

Fungal species	Disease / Host	Distribution	References
Metasphaeria anisometra	Twigs of *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Nattrassia mangiferae	Stem canker of *Eucalyptus* spp.	India	Sharma *et al.*, 1985
Pestalotiopsis versicolor	Stem (twig) canker of *Eucalyptus* spp.	India	Sharma *et al.*, 1985
Physalospora eucalyptorum	Shoot dieback & seedling blight, of *E. citriodora, E. ficifolia* & *E. salicifolia*	Italy	Spaulding, 1961
Rosellinia radiciperda	Root rot of *Eucalyptus* sp.	New Zealand	Spaulding, 1961
Ramularia pitereka (= *Sporotrichum destructor*)	Stem canker of *E. maculata*	Australia	Walker & Bertus, 1971
	Eucalyptus ficifolia	Australia	Smith, 1970
Seiridium eucalypti	Stem canker of *E. delegatensis*	Australia	Yuan & Old, 1995
Septobasidium curtisii	Stem canker of *Eucalyptus* sp.	USA	Westcott, 1971
Sphaeropsis sp.	Dieback, stem gall of *E. robusta, E. viminalis* & *Eucalyptus* sp.	USA	Farr *et al.*, 1989
Sphaeropsis tumefaciens	Dieback, stem gall of *E. cinerea*	USA	Farr *et al.*, 1989
Thyronectria pseudotricha	Stem canker of *Eucalyptus* spp.	India	ICFRE, 1990
Valsa eucalypti (anamorph *Cytospora eucalypti*)	Stem canker of *E. grandis* & *E. torelliana*	India	Sharma *et al.*, 1989
Valsa eucalypticola (anamorph *Cytospora eucalypticola*)	Stem canker of *Eucalyptus* spp.	India	ICFRE, 1990
	Stem canker of *Eucalyptus* spp.	Australia	Old *et al.*, 1986

CHAPTER 1: FUNGI ASSOCIATED WITH STEM CANKERS OF EUCALYPTS IN TASMANIA

1.1 INTRODUCTION

In order to better evaluate the range of eucalypt stem canker fungi present in Tasmania, a systematic survey was conducted in both plantations and natural forests.

Between May 1995 and April 1996, several survey trips were conducted, mainly to the northwest, northeast and the centre regions of Tasmania where eucalypt plantations have been established. More than sixty localities were visited (Fig. 1.1-1).

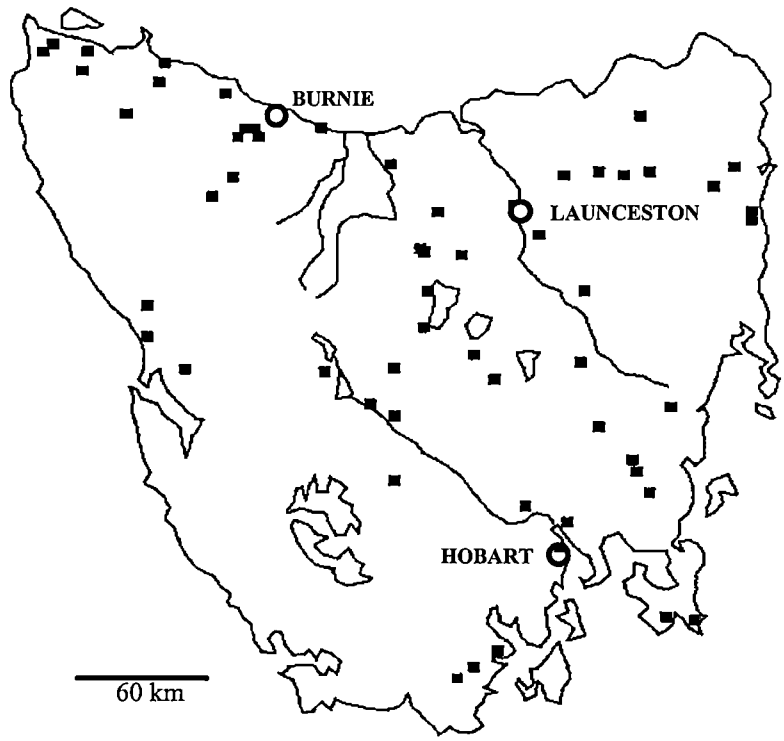


Fig. 1.1-1: Main collection points of fungi on eucalypts in Tasmania

Criteria of fungal classification, especially at the generic level are not well defined. It is necessary to follow a certain outline of classification that has been commonly used by most mycologists. In this survey, the systematic classification of ascomycota

follows "*Ainsworth & Bisby's dictionary of the fungi*" (8th edition) (Hawksworth *et al.* 1995). Anamorphic fungi belonging to Coelomycetes, Deuteromycotina based on the traditional classification of the fungi (Hawksworth *et al.* 1983) have no longer been treated as separate taxa and have been referred as to mitosporic fungi (Hawksworth *et al.* 1995). The fungal species investigated in this survey are divided into two sections, Ascomycotina and Mitosporic fungi. Under each section, they are arranged alphabetically in descriptive texts. For practical purposes, a key to species is provided to allow quick identification of the fungi found in the survey.

1.2 METHODS

1.2.1 FUNGAL COLLECTION

1.2.1.1 Sampling of trees

Sampling sites were selected on the basis of eucalypt stand types and species. Although the survey included roadside stands, woodland, natural forests and seed orchards (Table 1.1-1), emphasis was placed on sampling in eucalypt plantations, particularly plantations of *E. nitens*. Other eucalypt species investigated during the surveys were *E. amygdalina* Labill., *E. coccifera* Hook. f., *E. delegatensis* R. Baker, *E. globulus* Labill., *E. nitida* Hook. f., *E. obliqua* L'Hér., *E. ovata* Labill., *E. pauciflora* Sieber ex Sprengel, *E. pulchella* Desf, *E. regnans* F. Muell. and *E. rubida* Deane & Maiden.

Stems of eucalypts showing symptoms of shoot dieback, twig or stem lesions were collected. Particular attention was paid to fungal fruiting bodies on cankered branches. The health status of the trees at each site was noted, eg. if they were vigorously growing or stressed by insects, drought, etc.

In most cases, 50-100 trees within each stand were examined for presence/absence of stem canker fungi. However, the incidence of some canker diseases was recorded, eg. cankers associated with *Harknessia* cf. *eucalypti* Cooke in a 5-year-old plantation of *E. regnans*. In the plantation the percentage of trees bearing branch or stem cankers was calculated out of the total 100 trees in five rows (20/row) randomly investigated.

Table 1.1-1: Main eucalypt stand types sampled in the survey

Stand type	Number of locations visited	Age class	Eucalypt species
Plantations	1	4-year-old	*E. regnans*
	4	5-year-old	*E. nitens*
	2	5.5-year-old	*E. globulus, E. nitens*
	5	15-year-old	*E. globulus, E. nitens*
	5	4-6-year-old	*E. nitens, E. regnans*
	1	20-year-old (seed orchard)	*E. delegatensis*
Natural forests	22	>30-year-old	*E. amygdalina, E. delegatensis, E. nitida, E. obliqua, E. ovata, E. pulchella, E. viminalis, Eucalyptus* sp.
Woodlands	12	>20-year-old	*E. amygdalina, E. coccifera, E. globulus, E. pauciflora, E. tenuiramis*; *Eucalyptus* sp.
Roadside stands	5	>30-year-old	*E. amygdalina, E. obliqua, Eucalyptus* sp.

1.2.1.2 Isolation of canker fungi

Isolations of fungi were made from cankered tissue and fungal fructifications following the procedures previously described by Old *et al.* (1986). Cankers were sampled at three points along their longitudinal axis *ie.* at the top, middle and bottom of a lesion. Small pieces of wood or bark were cut from diseased tissue of the lesion and from apparently sound tissue adjacent to the lesion. The excised wood pieces were rinsed with 95% ethanol and washed with sterile distilled water (SDW), then surfaced-sterilised with 5% sodium hypochlorite for 2 min., and finally rinsed with SDW. The surface-sterilised wood pieces were placed onto 3% malt extract agar (MEA) and incubated for fungal colony development.

Isolations were also obtained from single fungal spores, eg. ascospores and conidia. The spores were suspended in sterile distilled water on sterile slides and then spread on a thin layer of 2% water agar in Petri dishes. Individual spores were transferred by means of a transfer needle to potato dextrose agar (PDA).

1.2.1.3 Induction of fungal fruiting

For those cultures not showing sporulation under normal incubation conditions, isolates were grown on (a) 3% malt extract agar (MEA) in plastic Petri dishes and

(b) autoclaved wheat-rice mixed bran medium in glass flasks. Fruitbodies were induced by incubating the cultures under a diurnal cycle of 12 hours fluorescent light (36W White 1H, 'THORN Power Saver') and 12 hours darkness at 20 °C until sporulation.

1.2.2 FUNGAL IDENTIFICATION

1.2.2.1 Morphological examination

The fruit bodies of canker specimens and cultural isolates were examined microscopically in the laboratory. Microscopic examinations of the specimens were carried out on squash mounts and thin median sections. The materials were mounted in Shear's mounting fluid and/or lactophenol cotton blue. Fifty mature spores (conidia or ascospores) were measured, and the arithmetic means calculated. Identification of fungi to genus and species levels was based on the relevant literature.

Descriptions of new fungi were published in Yuan and Mohammed (1997b,c,d,e,f). Detailed descriptions of all fungal species found during the survey are compiled in section 1.4. All these descriptions were based on materials (specimens) collected by the author. Herbarium materials of fungal collections were deposited in the Herbarium of the Institute for Horticultural Development, Knoxfield, Australia (VPRI).

1.2.2.2 Colony growth rate

The growth rate of some newly published species at different temperatures was studied using a gradient incubator (Lindner & May Pty. Ltd). Isolates of each fungal species were inoculated on 3% MEA in plastic Petri dishes (9 cm diam.). Three dishes per isolate were incubated at 10, 15, 20, 25 and 30°C respectively. Radial growth of colonies was measured daily. Average colony diameters were calculated after 25 days incubation (as most fast growing isolates were about to cover the whole dish).

1.2.2.3 DNA analysis

To confirm the separate identity of *Seiridium* species isolated from eucalypts, namely *S. eucalypti* and *S. papillatum*, in comparison to other *Seiridium* species on cypress, a preliminary molecular assay using ITS1 and ITS4 PCR amplication of rDNA

followed by RFLP analysis. The details of methods for DNA extraction, PCR amplification and RFLP analysis are given in Chapter 3.

1.3 SURVEY RESULTS AND DISCUSSION

A total of 212 specimens (isolates and/or herbarium material bearing fungal fruiting bodies) representing 30 fungal species were obtained (Table 1.3–1).

Ten of the fungal species detailed in this survey were new descriptions and published in relevant journals in accordance to present rules for the description of new species (Yuan and Mohammed 1997b,c,d,e,f). These newly proposed species are *Bertia antennaroidea, Ciliosporella tuberculiformis, Cryptodiaporthe curvata, Diaporthe fusispora, Neoplaconema cymbiforme, Phomatospora macrospora, Seiridium papillatum, Therrya eucalypti, Wuestneia campanulata* and *Wuestneia epispora.*

Five were new records for Australia: *Camarosporium propinquum* (Sacc.) Sacc., *Coniochaeta pulveracea* (Ehrh. : Fr.) Munk, *Eutypa spinosa* (Pers.:Fr.) Tul. & C. Tul., *Fenestella media* Tul. & C. Tul. and *Karstenula ceanothi* (Dearness & House) Barr. Eight were first reports on eucalypts (Table 1.3-1).

The most geographically ubiquitous fungal species found in the survey are shown in Figure 1.3–1. Of the thirty fungi obtained, *E. gyrosa*, *C. eucalypticola* and *Valsa* sp. were most frequently encountered, constituting 23.6%, 20.3% and 12.7% respectively of the total number of specimens (Table 1.3–1). Collections were made from 14 eucalypt species, with the large majority of samples originating from *E. nitens*, *E. regnans*, *E. delegatensis* and *E. obliqua*.

The results of the survey reflect the emphasis toward surveying plantations. Sixty of the specimens (comprising 12 different fungal species) were collected from nine *E. nitens* plantations, 31 (comprising 10 fungal species) from five *E. regnans* plantations, 17 (comprising 13 fungal species) from 3 *E. delegatensis* plantations and 16 (comprising 7 fungal species) from 7 *E. obliqua* plantations.

Table 1.3–1: Fungi associated with stem cankers of *Eucalyptus* spp. in natural forests and plantations of Tasmania during May 1995 – April 1996

Fungal species	Host[A]	Stand type[B]	No. of Collection	%[C]
Aulographina eucalypti	5	1	5	2.4
Bertia antennaroidea[D]	1, 4, 5, 8, 11,	1, 2,	5	2.4
Camarosporium propinquum[EF]	3, 9	1, 2, 3,	3	1.4
Ceuthospora innumera	5	1	2	1.0
Ciliosporella tuberculiformis.[D]	11	2	1	0.5
Coniochaeta pulveracea[EF]	3	2	1	0.5
Cryptodiaporthe curvata[D]	3	1	2	1.0
Cryptosporiopsis sp.	3	1	1	0.5
Cytospora eucalypticola	1-3, 5-11, 13	1, 2, 3, 4,	43	20.3
Diaporthe fusispora[D]	4, 5, 9	1, 3	4	1.9
Dichomera eucalypti	13	4	1	0.5
Dinemasporium strigosum[F]	5	1	1	0.5
Endothia gyrosa	1, 3-7, 10, 11, 13-15	1, 2,3,	50	23.6
Eutypa spinosa[EF]	7	2	1	0.5
Fenestella media[EF]	3, 9	2, 3	2	1.0
Karstenula ceanothi[EF]	5, 9, 13	1, 2, 3	3	1.4
Harknessia cf. *eucalypti*	11	1	4	1.9
Melanomma pulvis-pyrius[F]	11	1	1	0.5
Neoplaconema cymbiforme[D]	5	1	1	0.5
Pestalotiopsis neglecta	5	1	12	5.7
Phoma sp.	12	1 (?)	1	0.5
Phomatospora macrospora.[D]	3, 4	1	2	1.0
Seiridium eucalypti	1, 6, 7, 11	1,2	4	3.3
Seiridium papillatum[D]	3, 13	1, 2	2	1.0
Therrya eucalypti[D]	11	1	4	2.0
Thyrostroma eucalypti	1, 3, 11, 13	1, 2, 3,	6	2.9
Valsa ceratosperma	1, 3-7, 10, 11, 13, 15	1, 2, 3, 4	27	12.7
Wuestneia espora[D]	3, 5, 7, 11	1,3	7	3.3
Wuestneia campanulata.[D]	1, 4, 5, 8, 13	1, 2, 3	7	3.3
Zythiostroma sp.[F]	3, 5-8, 13	1, 2, 3, 4	8	3.8

[A] Host 1= *E amygdalina* Labill.; 2= *E. coccifera* Hook. f.; 3= *E. delegatensis* R. Baker; 4= *E. globulus* Labill.; 5= *E. nitens* (Deane & Maiden) Maiden; 6= *E. nitida* Hook. f.; 7= *E obliqua* L'Hér.; 8= *E ovata* Labill.; 9= *E. paucıflora* Sieber ex Sprengel; 10= *E. pulchella* Desf.; 11= *E. regnans* F. Muell.; 12= *E rubida* Deane & Maiden; 13= *Eucalyptus* sp.; 14= *E. tenuiramis* Miq.; 15= *E. viminalis* Labill.
[B] Stand type 1 = plantations; 2 = natural forests; 3 = woodland; 4 = roadside stands.
[C] Percentage of the total collections (isolations) for each fungal species
[D] New species published in Yuan and Mohammed (1997b,c,d,e,f)
[E] New record for Australia
[F] New record on eucalypts

Endothia gyrosa, along with its *Endothiella* anamorph was ubiquitous throughout Tasmania on different eucalypt species in plantations and native forests. It was mainly associated with annual stem cankers but diffuse cankers were also observed (Fig. 1.3-2*a, b, j, n, p & q*).

Although widespread in south eastern Australia, the teleomorph of *E. gyrosa* has never been found in Western Australia (Shivas 1989; Shearer 1994). In this survey, the teleomorph was found more frequently than the anamorph. Out of a total of 31 specimens with *E. gyrosa* fruit bodies, 20 (including 12 collected from plantations) were observed with the teleomorph and 11 (including 7 collected from plantations) with the anamorph. In three localities (one plantation and two native open woodland sites) both the anamorph and teleomorph were found in proximity on both the same and different trees.

This fungus was not usually associated with a high incidence of disease within stands of vigorously growing eucalypts except for a 16-year-old mixed provenance plantation of *E. nitens* at high quality site near Tewkesbury, northwestern Tasmania. In this one case, cankers caused by *E. gyrosa* were numerous and severe. Wardlaw (1998) has quantified disease severity at this site.

Endothia gyrosa cankers were found associated with visibly stressed trees. For example, in a 5-year-old plantation of *E. nitens* in north Tasmania, trees were heavily defoliated by autumn gum moth (*Mnesampela privata* Guen.) (Fig. 1.3-2*o*). Many of the trees had died and had been slashed. These dead trees were covered with fruiting bodies of *E. gyrosa*. Those trees still standing and exhibiting slight insect attack appeared to be dying from active cankers caused by *E. gyrosa* (Fig.1.3-2*p, q*) which were girdling the stems. The observations support the hypothesis of Old *et al.* (1990). They found under experimental conditions, *E. gyrosa* was more aggressive when trees were stressed by other environmental factors, such as defoliation.

Cytospora eucalypticola was found to be non-aggressive which confers with reports by Davison and Tay (1983), Old *et al.* (1986) and Shearer *et al.* (1987). It was

common on trees in open woodland areas (Fig. 1.3-2*k*), roadside stands and native forests, especially where crown dieback indicated that the trees were subject to stress. In a stand of mixed eucalypt species (*ca.* 30-year-old *E. pulchella* and *E. globulus*), coppice stems, produced after fire damage, bore abundant fruit bodies of *C. eucalypticola* and also a *Valsa* species (Fig. 1.3-2*m*). The latter has not been previously reported in Tasmania and is probably the teleomorph of *C. eucalypticola* (Old *et al.* 1991). It was frequently associated with dead branches in the lower (shaded) crown of *E. nitens* and *E. globulus* in young plantations throughout Tasmania.

Aulographina eucalypti, a common leaf pathogen (Wall and Keane 1984) was often found on shaded dead and dying branches in plantations of *E. nitens*.

Ciliosporella tuberculiformis was collected in two different areas at a single locality. It was associated with distinctive diffuse cankers on branches of *E. regnans* showing tip dieback (Fig.1.3-2*c*).

Harknessia cf. *eucalypti* was associated with severe branch and stem cankers in a 5-year-old *E. regnans* plantation at Westfield (Fig.1.3-2*d*, *e* & *l*). An investigation of disease incidence showed that out of a total of 100 trees examined, 25 had branch cankers and six had main-stem cankers. Cankers were diffuse with large swollen areas and cracked bark. They contained abundant, cream coloured wart-like fruit bodies. As these fruit bodies matured they became black and powdery on the top (Fig.1.3-2*d* & *e*). Most of the known species of *Harknessia*, including *H. eucalypti* have been found on leaves or occasionally on small dead twigs (Sutton 1980; Nag Raj 1993). This is the first record of this fungus being associated with severe stem and branch canker symptoms in a eucalypt plantation. A teleomorph of the *Harknessia* fungus, *Wuestneia epispora*, was collected from several locations on *E. delegatensis, E. nitens*, *E. obliqua* and *E. regnans*.

Seiridium eucalypti was first isolated from *E. delegatensis* in Tasmania in 1987 (Yuan and Old 1995) and was collected on several occasions during this survey. Fruiting

bodies of this fungus were collected from a dead branch of *E. amygdalina* with no apparent canker symptoms. Several other collections of *S. eucalypti*, however, were associated with stem canker symptoms (Fig.1.3-2*f* & *g*); one from native *E. obliqua* in northern Tasmania, two from a 5-year-old *E. regnans* plantation at Westfield, south central Tasmania and three from a hybrid *of E. camaldulensis* Dehnh. and *E. nitens* in northern Tasmania. The canker symptoms observed in the field on stems of *Eucalyptus* spp. were very similar to those produced by artifical inoculation of 12-month-old eucalypt seedlings with isolates of *S. eucalypti* (Yuan and Old 1995; Yuan, Chapter 2).

Species of the genus *Phoma*, eg. *P. eucalyptica* Sacc. have been reported as eucalypt stem canker agents (Azevedo 1971; Gibson 1975). In this survey a single collection was made of a *Phoma* species which was associated with a large (*ca.* 15 cm in diam.), sunken annual stem canker on *E. rubida* Deane & Maiden.

Botryosphaeria dothidea (syn. *B. ribis* Grossenb. & Dugg.) was not found in this survey although it has been reported elsewhere in Australia as a common canker agent (Davison and Tay 1983; Shearer *et al.* 1987; Old *et al.* 1990). In their study on fungal endophytes in leaves, xylem and bark of *E. nitens* in Australia, Fisher *et al* (1993) also failed to find *B. dothidea.* However, this fungus was present on *E. nitens* in the UK (Fisher *et al.* 1993). In South Africa, *B. dothidea* has been commonly observed occurring as an endophyte in healthy leaves of *E. grandis* W. Hill ex Maiden and *E. nitens* and to be associated with twig die-back and stem cankers on various eucalypt species including *E. macarthurii* Deane & Maiden, *E. grandis* , *E. nitens, E. smithii* R. Baker and *E. urophylla* S.T. Black (Smith *et al.* 1994).

Recently, in Western Australia, a species of *Zythiostroma* was isolated from cankers on *Banksia coccinea* R. Brown and has demonstrated a strong pathogenic ability in artificial inoculation tests (Shearer *et al.* 1995). *Zythiostroma* sp. was found on several different eucalypt species in this survey, but the fungus was associated with stem canker symptoms in only three out of eight collections (Fig.1.3-2*h* & *i*). The other five samples collected were fruit bodies on dead tissue.

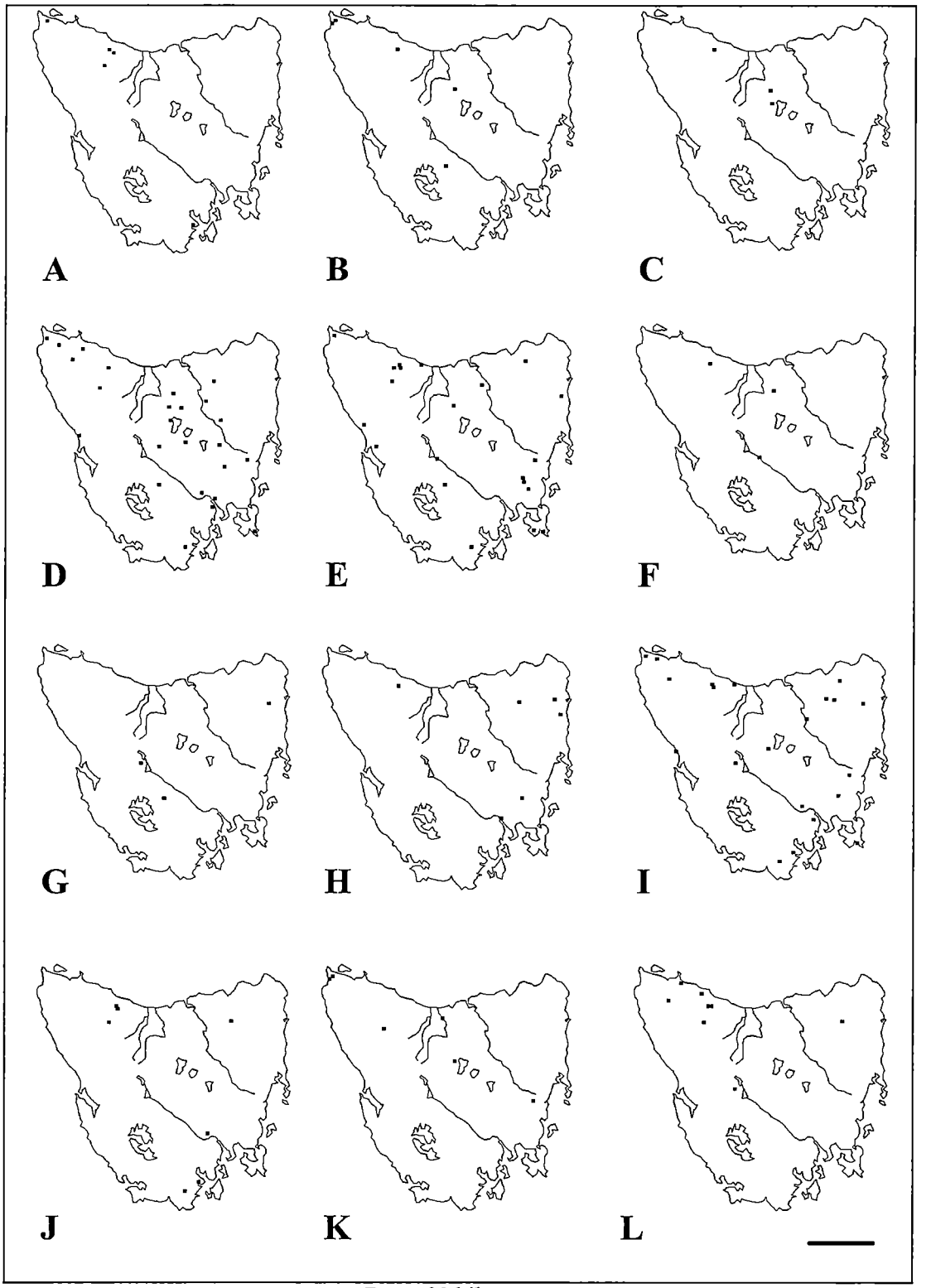


Fig. 1.3–1: Collection localities (dots) of the most geographically ubiquitous fungal species found in the survey

A. *Aulographina eucalypti*; **B.** *Bertia antennaroidea*; **C.** *Camarosporium propinquum*; **D.** *Cytospora eucalypticola*; **E.** *Endothia gyrosa*; **F.** *Karstenula ceanothi*; **G.** *Seiridium eucalypti*; **H.** *Thyrostroma eucalypti*; **I.** *Valsa ceratosperma*. **J.** *Wuestneia epispora*; **K.** *Wuestneia campanulata*; **L.** *Zythiostroma* sp.

Fig. 1.3–2: Canker symptoms on eucalypts in Tasmania

A. Branch canker of 5-year-old *Eucalyptus regnans* associated with *Endothia gyrosa*;

B. Fruiting bodies (teleomorph) of *E. gyrosa*; tangential section (arrow) through stromata showing orange-coloured stromata and embedded perithecia (black dots);

C. Diffuse branch canker of 6-year-old *E. regnans* associated with *Ciliosporella tuberculiformis* (the fruitbodies are only in necrotic tissue of canker);

D & E. Stem cankers of 5-year-old *E. regnans* associated with *Harknessia* cf. *eucalypti*;

F. Branch canker of 5-year-old *E. regnans* associated with *Seiridium eucalypti*;

G. Branch canker of 30-year-old *E. obliqua* associated with *S. eucalypti*;

H. Branch canker of 30-year-old *E. ovata* associated with *Zythiostroma* sp.;

I. Branch canker of 30-year-old *E. obliqua* associated with *Zythiostroma* sp. (note black dots; conidiomata);

J. Annual canker produced by *Endothia gyrosa* associated with small branch stub on 16 year old *Eucalyptus nitens* (note orange conidiomata);

K. Diffuse canker on branch of 30-year-old *E. coccifera* from which *Cytospora eucalypticola* was isolated;

L. Annual canker on main stem of a 30-year-old *E. regnans* tree from which *Harknessia* cf. *eucalypti* was isolated;

M. A fire stressed tree of *E. globulus* (30-year-old); stem was covered with numerous conidiomata of *C. eucalypticola*;

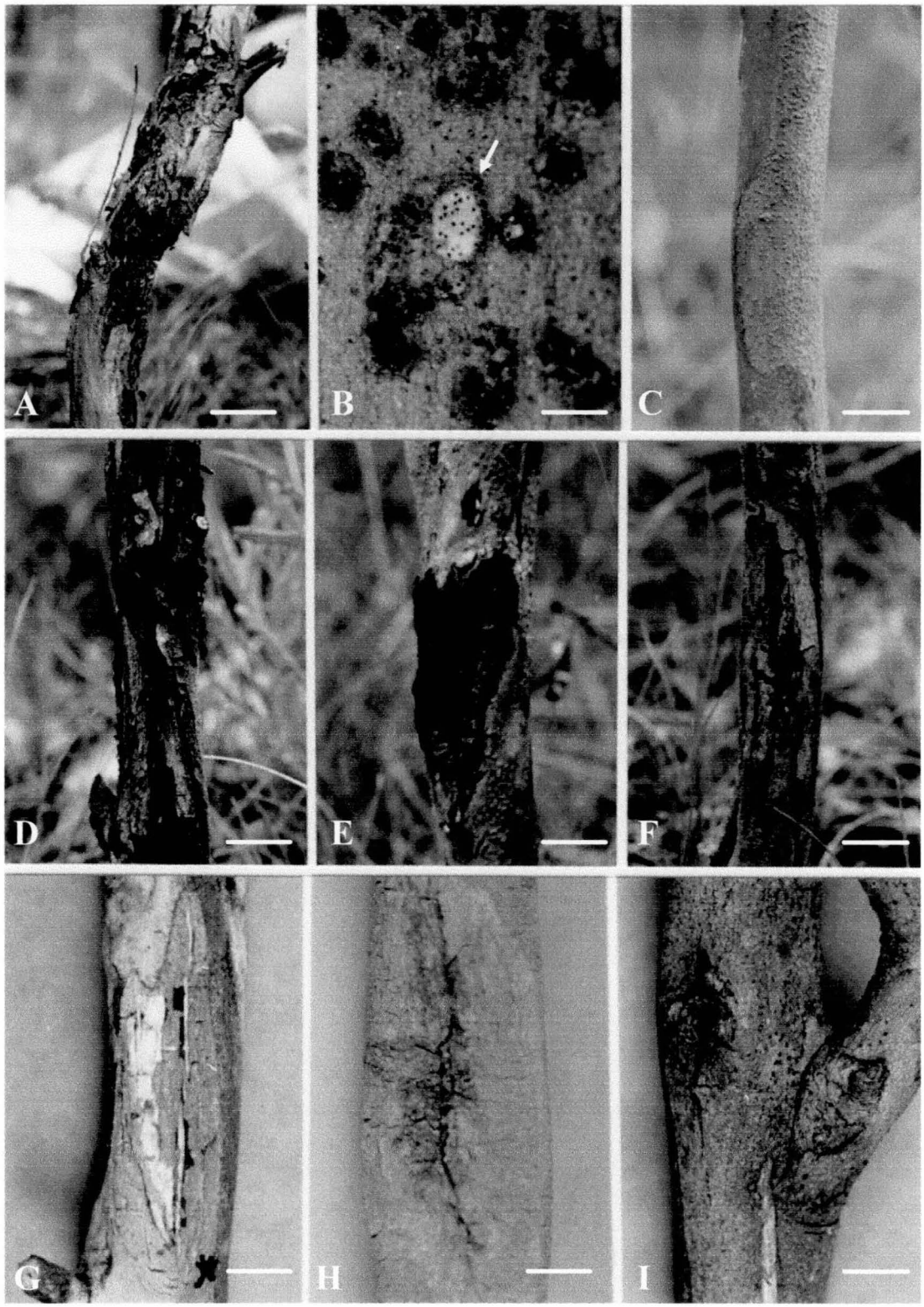
N. A 16-year-old rough-barked tree of *E. nitens* showing bark infected by *E. gyrosa* at Ridgley, north Tasmania;

O. 5-year-old *E. nitens* heavily defoliated by autumn gum moth (*Mnesampela privata* Guen.);

P. Upper stem of a defoliated tree showing infection by *E. gyrosa*;

Q. Detail of Fig. G showing the canker extending down the stem.

Fig. 1.3–2: Canker symptoms on eucalypts in Tasmania



Bar = 1.5 mm for ***B***; 10 mm for ***H***; 20 mm for ***A***, ***C-G*** and ***I***

Fig. 1.3–2: Canker symptoms on eucalypts in Tasmania (continued)

Bar = 6 cm for ***J***; 4cm for ***K*** & ***Q***; 10cm for ***L***;
= 100 cm for ***M***; 50cm for ***N*** & ***O***; 20 cm for ***P***

1.4 TAXONOMIC DESCRIPTION OF SPECIES

1.4.1 ASCOMYCOTA

1. ***Aulographina eucalypti*** (Cooke & Massee) von Arx & Müller
Sydowia 14: 331 (1960)

Syn.: *Aulographum eucalypti* Cooke & Massee, Grevillea 18: 6 (1889)
Lembosiopsis eucalyptina Petr. et Syd., Ann. Mycol. 22: 372 (1924)

Ascomata hysterothecioid, superficial, elongate, sometimes branched or star-like. Asci bitunicate, ovate, sessile, thick towards the apex, 8-spored, 20–30 x 9–11 μm. Ascospores hyaline, ellipsoid, 2-celled, constricted at the septum, asymmetric, upper cell wider but shorter than the lower cell, 8–11.5 x 3.5–5 μm.

On dead and dying twigs and branches of eucalypts.

Specimens examined: Tasmania: Blue Gum Rd, Ridgley, on *Eucalyptus nitens*, 1 Aug. 1995, *Z.Q. Yuan* 69b; Goulds, Dover, same host, 11 Aug. 1995, *Z.Q. Yuan* 63; West Ridgley, same host, 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W16c; Wages Rd, Surrey Hill, same host, 18 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W13c Woolnorth, same host 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W12b.

Aulographina eucalypti has frequently been found on a variety of eucalypt species in both natural forests and plantations. It is distributed throughout most regions of Australia (Wall and Keane 1984; Sankaran, Sutton and Minter 1995; Yuan 1996).

2. ***Bertia antennaroidea*** Z.Q. Yuan (Figs.1.4–1a,b; 1.4–2a,b; 1.4–3a,b)
in Yuan and Mohammed, Mycotaxon 63: 10 (1997)

Ascomata perithecioid, superficial, separate to densely gregarious, seated on a subiculum composed of brown to dark brown septate and branched hyphae, black; the outer surface conspicuously marked with coarse warts, without ostioles, spherical, 250–500 μm diam. Peridium 35–65 μm wide, of dark, large, thick–walled

pseudoparenchymatous cells, 7–25 μm in diam. Asci 100–125 x 10–14 μm, clavate to subcylindrical, short stalked, thin-walled, with a nonamyloid apical ring, 8-spored. Ascospores 27.5–37.5 x 4.5–7.0 (mean 35 x 6.0) μm, biseriate at the upper parts or obliquely monoseriate, hyaline, 1-septate, elongated ellipsoid, straight or curved, slightly constricted at the septum, smooth-walled, surrounded with a thin mucilaginous sheath.

On dead stems or decorticated branches of *Eucalyptus* spp.

Specimens examined: Tasmania: Welcome River, Smithton, on *E. ovata*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC37a (VPRI 21064, Holotype); Florentine Valley, Westfield, on *Eucalyptus regnans* F. Muell., 13 July 1995, *Z.Q. Yuan* 39 (VPRI 21065); Jacky Marsh Rd, Poatina, on *E. amygdalina* Labill., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET34 (VPRI 21066); Woolnorth, on *E. globulus* Labill., 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W11a (VPRI 21067); West Ridgley, on *E. nitens* (Deane & Maiden) Maiden, 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W22a (VPRI 21068).

Several characteristics distinguish *B. antennaroidea* from other species of *Bertia*. These include: the presence of unique subicules at bases of ascomata; asci with apical rings; and ascospores which are always 1-septate, constricted at the septum and surrounded by a thin gelatinous sheath.

The presence of hyphal subicules at the ascoma base and the gelatinous sheaths surrounding ascospores in *B. antennaroidea* are features not previously observed in any known species of *Bertia* (Munk 1957; Corlett and Krug 1984; Subramanian and Sekar 1990; Hsieh *et al.* 1995; Hyde 1995). However, Krug and Corlett (1988) found a black basal subicule-like crust in *B. sinensis* Krug & Corlett. *B. sinensis* has geniculate ascospores which are different to those of *B. antennaroidea*.

The presence of the subicule in *B. antennaroidea* might suggest affinity to *Calyculosphaeria* Fitzpatrick. The latter genus, however, has small, fusiform, 1-septate ascospores and has been included as a synonym of *Nitschkia* Otth. (Hawksworth *et al.* 1995).

Previously, only one species of *Bertia* had been recorded in Australia. *Bertia convolutispora* K.D. Hyde was described from submerged wood in streams (Hyde 1995). It has little in common with *B. antennaroidea*, except for the presence of ascal apical rings.

3. *Coniochaeta pulveracea* (Ehrh.: Fr.) Munk (Figs.1.4–1c; 1.4–2i; 1.4–3e)

Dansk Bot. Ark. 17: 90 (1957)

Syn.: *Sphaeria pulveracea* Ehrh.: Fr.,
Rosellinia pulveracea (Ehrh.: Fr.) Fuckel,
Sphaeria obliquata Sommerf.,
Rosellinia obliquata (Sommerf.) Sacc.,

Ascomata perithecioid, superficial, gregarious, broadly ovate to subglobose, 200–300 μm wide, 250–350 μm high, ostiolate, glabrous, with pale brown hyphae at the base. Peridium up to 45 μm thick, composed of dark thick-walled pseudo-parenchymatous cells. Asci 80–110 x 10–13 μm, cylindrical, obtuse to truncate at apex, 8-spored. Ascospores 10–13 x 7–8 μm, ellipsoid to broad ellipsoid, brown to dark brown, smooth-walled, with a longitudinal germ slit.

On dead stems of eucalypts.

Specimen examined: Tasmania: Ben Ridge, St. Helens, on *Eucalyptus delegatensis* R. Baker, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET25a (VPRI 20829).

The morphology of this fungus on eucalypt is close to that of *C. ligniaria* (Grev.) Cooke. The latter differs from *C. pulveracea* in its perithecia covered with setae and its slightly larger ascospores (*fide* Munk 1957; Mahoney and LaFavre 1981). Several species of the genus *Coniochaeta* have been recorded previously in Australia (Hawksworth and Yip 1981).

4. *Cryptodiaporthe curvata* Z.Q. Yuan (Figs.1.4–1d; 1.4–2g,h; 1.4–3f,g; 1.4–9a,b)
in Yuan and Mohammed, Mycotaxon 63: 12 (1997)

Ascomata immersed, developing singly or in clusters of two to four within the periderm, appearing on the surface as numerous minute, papillate pustules. Stromatic tissue prosenchymatous, poorly developed, mixed with the host tissue. Perithecia spherical or with flattened bases, 250–350 µm wide, 250–400 µm high, black; ostiole lined with periphyses. Peridium 40–50 µm wide, consisting of several layers of dark, thick-walled rounded pseudoparenchymatous cells, 10–20 µm diam. Asci 62.5–87.5 x 12.5–17.5 µm, unitunicate, clavate, with a tapering base, with nonamyloid refractive rings at a thickened apex, 8-spored. Ascospores 20–35 x 5–7.5 µm, obliquely or irregularly biseriate in the ascus, hyaline, oblong-ellipsoid to cylindrical, rounded ends, with one median septum, slightly constricted at the middle, inequilateral, surrounded by a thin gelatinous sheath.

On dead stems of eucalypts.

Specimens examined: Tasmania: Seed orchard, Ridgley, on *E. delegatensis*, 16 April 1996, *Z.Q. Yuan* W20b (VPRI 21069, holotype); same location and host, 16 April 1996, *Z.Q. Yuan* W21 (VPRI 21070).

The scattered, immersed ascomata with poorly developed stromata in *C. curvata* are similar to those of *C. aubertii* (West.) Wehmeyer. Furthermore, the ascospore size of *C. curvata* is close to that of *C. magnispora* (Ell. & Ev.) Wehmeyer. However, the latter species has even larger, ellipsoid to fusoid non-constricted ascospores (35–40 x 7–9 µm, *fide* Wehmeyer 1933). The only species closer to *C. curvata* in ascospore size and shape is *C. salicella* (Fr.) Petr., which has ascospores measuring 14.2–27.8 x 4.4–7.7 µm (*fide* Mathiassen 1993). As illustrated by Mathiassen (1993), quite a few of the ascospores of *C. salicella* in the type material were oblong-ellipsoid with rounded apices and were often slightly bent as in *C. curvata*. Despite the similarities, the constriction in the middle of the ascospores of *C. curvata* appears to be a unique character.

The inequilateral, oblong-ellipsoid to cylindrical ascospores with narrower middles of *C. curvata* may suggest affinity with *Vialaea* Sacc.. However, species of *Vialaea* have ascomata scattered singly without stromatic tissue and asci with amyloid apical rings. Ascospores of *Vialaea* are strongly constricted in the middle (propeller-shaped) and the genus was included in the Amphisphaeriaceae by Müller and Arx (1973). Cannon (1995) re-studied the genus *Vialaea* and established the family Vialaeaceae to accommodate this genus.

Species of the genus *Cryptodiaporthe* have not been previously recorded on eucalypts (Sankaran *et al.* 1995). Recently a species, *C. melanocraspeda* Bathgate, Barr & Shearer was described on *Banksia coccinea* R. Br. from Western Australia, but it has little in common with *C. curvata* (Bathgate *et al.* 1996).

5. *Diaporthe fusispora* Z.Q. Yuan (Figs.1.4–1e,f; 1.4–2e,f; 1.4–3d)
in Yuan and Mohammed, Mycotaxon 63: 14 (1997)

Ascomata separate or loosely clustered, immersed beneath a dorsal zone along a blackened bark surface, globose or subglobose, 225–300 μm wide, 200–250 μm high; beak protruding through bark, 600–900 μm high and 100–120 μm diam at base, 60–80 μm at apex. Peridium 17–22 μm wide, composed of several layers of compressed cells. Asci 37.5–43 x 7.5–10 μm, clavate, with nonamyloid refractive apical rings, 8-spored. Ascospores 10–15 x 3–5 (mean = 12 x 4.2) μm, overlapping biseriate, hyaline, fusoid, sometimes elliptic-fusoid, straight or slightly curved, with 1 median septum, constricted at the septum, bearing a delicate setose appendage at each end, 1–1.5 μm long; 4-guttulate.

On stems of eucalypts.

Specimens examined: Tasmania: West Ridgley, on *E. globulus*, 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W15 (VPRI 21071, Holotype); same location and host, 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W17 (VPRI 21072); same location, on *E. nitens,* 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W22d (VPRI 21073); Porter Bridge Rd, Deloraine, on *E. pauciflora* Sieber ex Sprengel, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC66b (VPRI 21074).

The ascoma habit and size of ascospores, as well as the presence of the delicate ascospore appendages in this fungus are similar to those of *D. eucalypticola* K.M. Old & Z.Q. Yuan described on *Eucalyptus* spp. in Australia (Yuan *et al.* 1995). However, the ascospores of *D. eucalypticola* are elongate-ellipsoid to cylindrical and not constricted at the septum, while those of *D. fusispora* are fusoid in shape and constricted at the septum. *D. medusaea* Nit. (syn. *D. eucalypti* Harkn.) another species previously recorded on eucalypts, has ascospores which are similar in shape to those of *D. fusispora*. However *D. medusaea* differs in its collectively erumpent ascomata and lack of appendages on the ascospores. Furthermore, its ascospores are narrower (2.5–3.5 μm wide, *fide* Wehmeyer 1933).

6. *Endothia gyrosa* (Schwein. : Fr.) Fries (Figs.1.3–2b; 1.4–10d)

Summa Veg. Scand., p. 385 (1849)

Syn.: *Sphaeria gyrosa* Schw. ex Fries, Syst. Mycol. 2: 419 (1823)

Melogramma gyrosum (Schw. ex Fries) Tul. in Cooke, Ann. New York Acad. Sci. 1: 185 (1978)

Stromata erumpent through bark, 0.5–3 mm wide and 1–2 mm high, bright orange internally and externally, but becoming rusty brown on the stem surface with age. Perithecia deeply immersed in stroma with elongate beaks emerging at surface of stroma as black dots, subglobose, 100–300 μm diam, dark brown to black. Asci unitunicate, subclavate to fusoid, 20–35 x 4–7 μm, 8-spored. Ascospores hyaline, one-celled, fusiform to cylindrical, slightly curved, 5.5–12.0 x 1.0–2.0 μm.

On cankered stems of eucalypts.

Specimens examined: Tasmania: Black Jack's Hill, Little Seanport, on *E. amygdalina,* 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET7a; Florentine Valley, Westfield, on *Eucalyptus regnans* F. Muell., 13 July 1995; same location and host, 15 Feb. 1996, *Z.Q. Yuan* 94,95; same location and host, 22 Sept. 1996, *Z.Q. Yuan* 295; Jackeys Marsh, on *E. delegatensis*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET35; Mara Creek, Branxholm, on *E. regnans*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET29; Mitchells Creek, Buckland, on *E. globulus*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET1; Mossy Marsh, on *E. obliqua*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET52; Srahan, on *E. nitida*, 11 Dec. 1995, *Z.Q. Yuan*

& M. Hall WC10; St. George Rd, Ridgley, on *E. nitens*, 17 Dec. 1995, *Z.Q. Yuan & M. Hall* W18; Trafalgar Flant, St. Helens, on *E. viminalis*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET17a; Yorkys Creek, Scamander, on *E. regnans*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET9; Zeehan, on *E. nitida*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC18, WC21

7. *Eutypa spinosa* (Pers.: Fr.) Tul. & C. Tul. (Figs.1.4–1g; 1.4–2j,k)

Sel. Carp. Fung. 2: 59 (1863)

Syn.: *Sphaeria spinosa* Pers.: Fr., Pers. in Röm., Neu Mag. Bot. 1: 83 (1794), Syst. mycol. 2: 368 (1823)

Valsa spinosa (Pers.: Fr.) Nitschke, Pyrenomyc. germ. 1: 127 (1867)

Diatrype berengeriana De Not., Sfer. ital. 1: 27 (1863)

Stroma long and effused, up to 130 mm long and 10 mm wide, brown to dark brown, punctulate. Perithecia immersed in stromata in a single layer with ostiolar necks separately erumpent (eutypoid); ostioles conical, 3–5 sulcate (mostly 3). Asci unitunicate, cylindrically clavate or spindle-shaped, long-stipitate, with visible amyloid apical rings, 8-spored, 25–35 x 4–6 μm (p. sp.); pedicels 30–80 μm long. Ascospores 1-celled, allantoid or suballantoid, pale brown, 6–8 x 1.8–2.5 μm.

On stems of eucalypt.

Specimen examined: Tasmania: Mawbanna Rd, Mawbanna, on *E. obliqua* L'Hér., 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC56 (VPRI 21075).

Four species of *Eutypa* have been previously recorded in Australia, of which *E. lata* (Pers.: Fr.) Tul. & C. Tul. and *E. armeniacae* Hansf. M.V. Carter were reported from Tasmania; *E. lata* on the wood and bark of unnamed plants and *E. armeniacae* on *Prunus armeniaca* L. (Cooke 1892; Sampson and Walker 1982). However, *E. armeniacae* is considered to be conspecific with *E. lata* by Rappaz (1987). Based on the report of Rappaz (1987), the present fungus on eucalypts easily fits the morphological description for *E. spinosa*. The fungus was originally described from wood of *Fagus sylvatica* in Europe. This is the first record of this species from Australia.

8. *Fenestella media* Tulasne & C. Tulasne (Figs.1.4–1h; 1.4–2d; 1.4–3j)

Sel. Carp. Fung. 2: 207. 1863

Ascomata immersed in valsoid groups 1–2 mm wide or in elongated rows 3–4 mm long, 300–500 μm diam. Peridium brown, 30–50 μm thick, of brown, thick-walled pseudoparenchymatous cells 10–12 μm in diam. Asci 100–125 x 18–22.5 μm, clavate, 8-spored. Ascospores irregularly biseriate, 27–40(–52.5) x 10–18 (mean 36.2 x 13.0) μm, yellowish brown to dark brown, end cells pale, ellipsoid-fusoid, ends somewhat acute, 5–13 septa, 3–5 longitudinal septa, constricted at first-formed septum, wall dark, smooth.

On stems of *Eucalyptus* spp.

Specimens examined: Tasmania: Ben Ridge, St. Helens, on *Eucalyptus delegatensis*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET25b (VPRI 20830); Porter Bridge Rd, Deloraine, on *E. pauciflora*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC66c (VPRI 20831).

Morphologically, the fungus reported here fits the description of *F. media* with the exception that the ascospores of *F. media* occasionally bear oblong terminal appendages (Barr 1990). This species has been found on branches of many hardwood species in temperate zones of the northern hemisphere, but has not been previously recorded in Australia on eucalypts.

9. *Karstenula ceanothi* (Dearness & House) Barr (Figs.1.4–1i; 1.4–2c; 1.4–3c)

North American Flora 13: 49 (1990)

Syn.: *Thyridium ceanothi* Dearness & House in House, Bull. New York State Mus. 179: 31 (1915)
Xylosphaeria ceanothi (Dearness & House) Petrak, Sydowia 4: 18 (1950)
Mycothyridium ceanothi (Dearness & House) Petrak, Sydowia 15: 189 (1962)

Ascomata gregarious or scattered, immersed in a thin blackened clypeus, spheroid, 300–500 μm wide, 200–300 high, with a short papillate apex. Peridium 25–35 μm wide, brown, of dark thick-walled elongated cells at outer part and hyaline compressed cells at inner part. Asci 100–130 x 10–12 μm, cylindrical, 8-spored. Ascospores 17.5–22.0 x 7.5–10 μm, uniseriate, brown to dark brown, with end cells

slightly paler, ellipsoidal to fusoid, with obtuse ends, symmetric, 3-septate, constricted at the first-formed septum, with a longitudinal septum in mid cells, often with one additional longitudinal or oblique septum at one or both end cells; wall smooth.

On dead stems of eucalypts.

Specimens examined: Tasmania: Wayatinah Rd, Tarraleah, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET55 (VPRI 21076); Porter Bridge Rd, Deloraine, on *E. pauciflora*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC66a (VPRI 21077); West Ridgley on *E. nitens,* 16 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W22b (VPRI 21078).

Almost all morphological features and measurements of ascomata, asci and ascospores in these collections on eucalypts conform to the description of *K. ceanothi* (Dearness & House) Barr, except that the blackened clypeus was observed only when the ascomata were immersed in groups. *Karstenula ceanothi* has only been reported previously from North America (Barr 1990) and this is the first record in Australia on *Eucalyptus* spp.

10. *Melanomma pulvis-pyrius* (Pers.: Fr.) Fuckel

Jahrb. Nassauischen Vereins Naturk. 23–24: 169 (1870)

Syn.: *Shpaeria pulvispyrius* Pers.:Fr., Syst. Mycol. 2: 458 (1823)

Melanomma subsparsa Fuckel, Jahrb. Nassauischen Vereins Naturk 23–24: 160 (1870)

Melanomma verrucaria (Fr.) Sacc., Syll. Fung. 2: 107 (1883)

(Other synonyms see Barr, 1990)

Ascomata perithecia, superficial, gregarious to crowded on blackened surface of branches, ellipsoid to globose with a short papillate-like apex, 250–550 µm, black, with rough surface. Asci bitunicate, cylindrical, 100–150 x 8–12 µm, 8-spored. Ascospores 17–21 x 4–6 µm, uniseriate, light brown to brown, ellipsoid to fusoid with ends acute, sometimes slightly curved, 3-septate, constricted at the middle septum.

On dead decorticated branches of eucalypts.

Specimens examined: Tasmania: Florentine Valley, Westfield, on *Eucalyptus regnans* F. Muell., 20 July 1995, *Z.Q. Yuan* 57.

This is a common and widespread species on dead stems of various trees and shrubs (Barr 1990), but has not been recorded on eucalypts before (Sankaran Sutton and Minter 1995).

11. *Phomatospora macrospora* Z.Q. Yuan (Figs.1.4–1j; 1.4–2l,m; 1.4–3h,i)
in Yuan and Mohammed, Mycotaxon 63: 17 (1997)

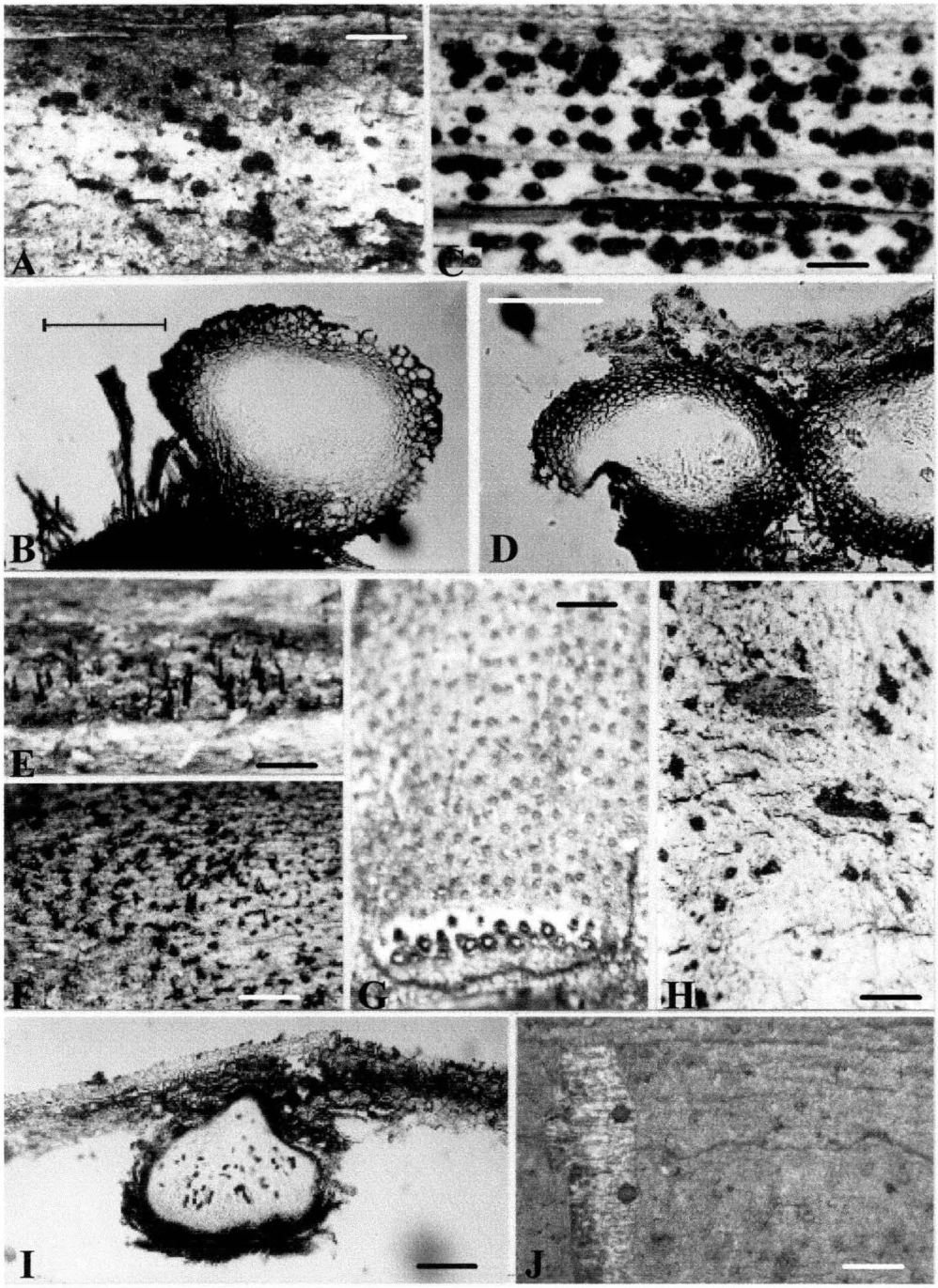
Ascomata perithecioid, scattered, deeply immersed, with a minute tip of the perithecial apex protruding through the surface of the host tissue, without stromatic tissue, spheroid to more or less triangular, 300–500 μm wide, 250–400 μm high. Peridium thin, 9–12 μm wide, composed of few layers of thin-walled, compressed pseudoparenchymatous cells, light brown to brown externally. Asci 162–213 x 12.5–15.5 μm, cylindrical, thin-walled, short-stalked, with a small apical nonamyloid ring, 8-spored. Ascospores 30–47.5 x 8.8–12.5 (mean 39.0 x 10.5) μm, irregularly or obliquely uniseriate, elliptic-cylindric to fusoid-cylindric, aseptate, hyaline, with one or two conspicuous oil drops at the centre or ends, granular, thin-walled, smooth.

On stems of eucalypts.

Specimens examined: Tasmania: Seed orchard, Ridgley, on *E. delegatensis*, 16 April 1996, *Z.Q. Yuan* W20a (VPRI 21079, holotype); Woolnorth, on *E. globulus*, 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W11b (VPRI 21080).

The genus *Phomatospora* Sacc. is characterised by having nonstromatic, thin-walled and noncarbonaceous perithecia which are immersed in host tissue and possess an apical ostiole with periphyses. Asci are unitunicate and formed in a basal layer. There is a faint refractive (nonamyloid) ring in the apex of an ascus.

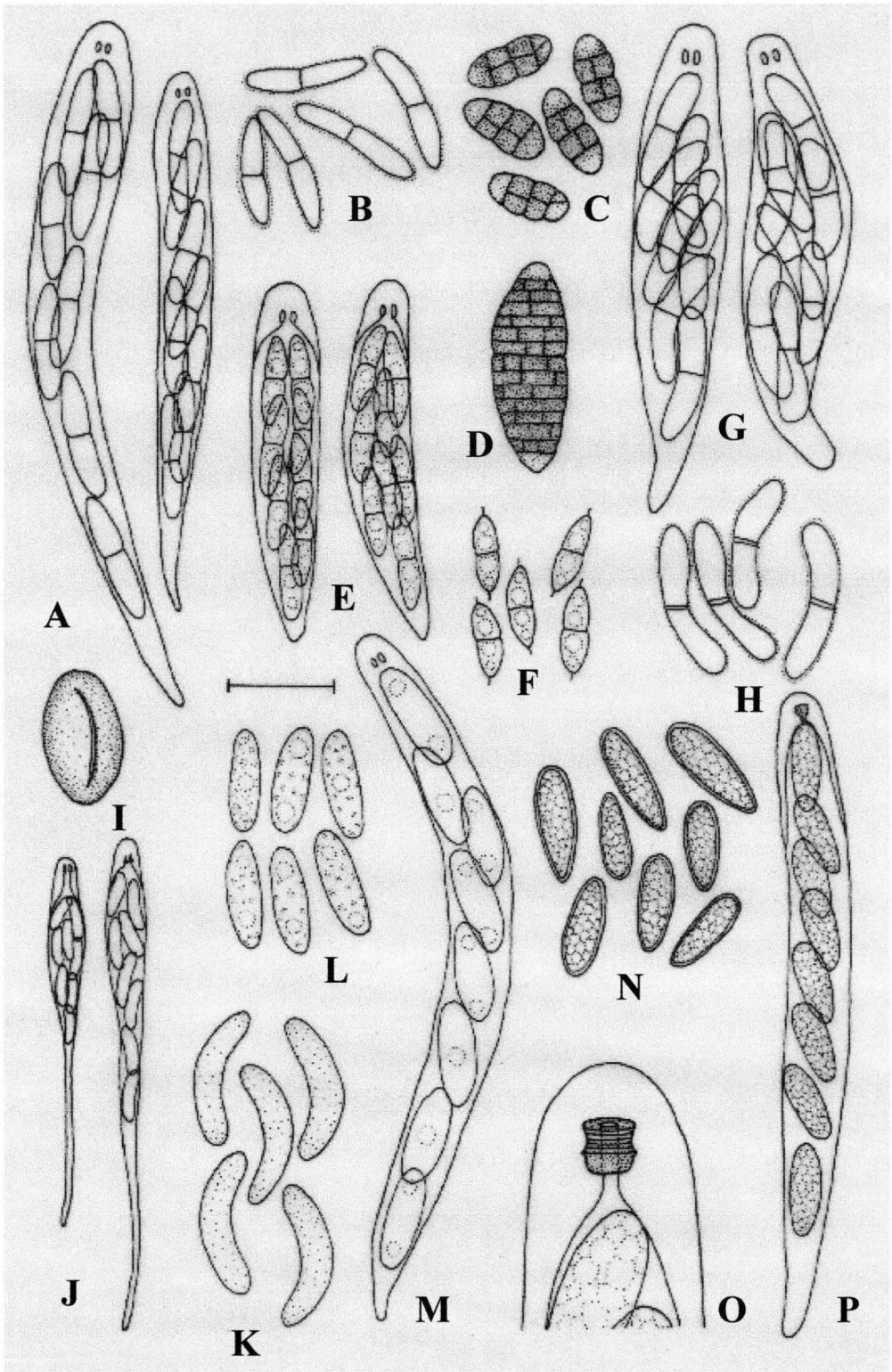
Most species of *Phomatospora* are found on herbaceous stems and grass culms (von Arx and Müller, 1954; Barr 1970; Wehmeyer 1975; Rappaz 1992). Only *P. dinemasporium* J. Webster, a common species found on many grass culms (Webster 1955), had been recorded on the wood of eucalypts (*Eucalyptus viminalis* in Argentina) (Romero 1983; Sankaran, Sutton and Minter 1995).



Bar = 120 μm for **B** & **D**; 120 μm for **I**; 1 mm for **C**; 1.5 mm for **A** & **J**; 2 mm for **E** & **F**; 2.5 mm for **G**; 3 mm for **H**

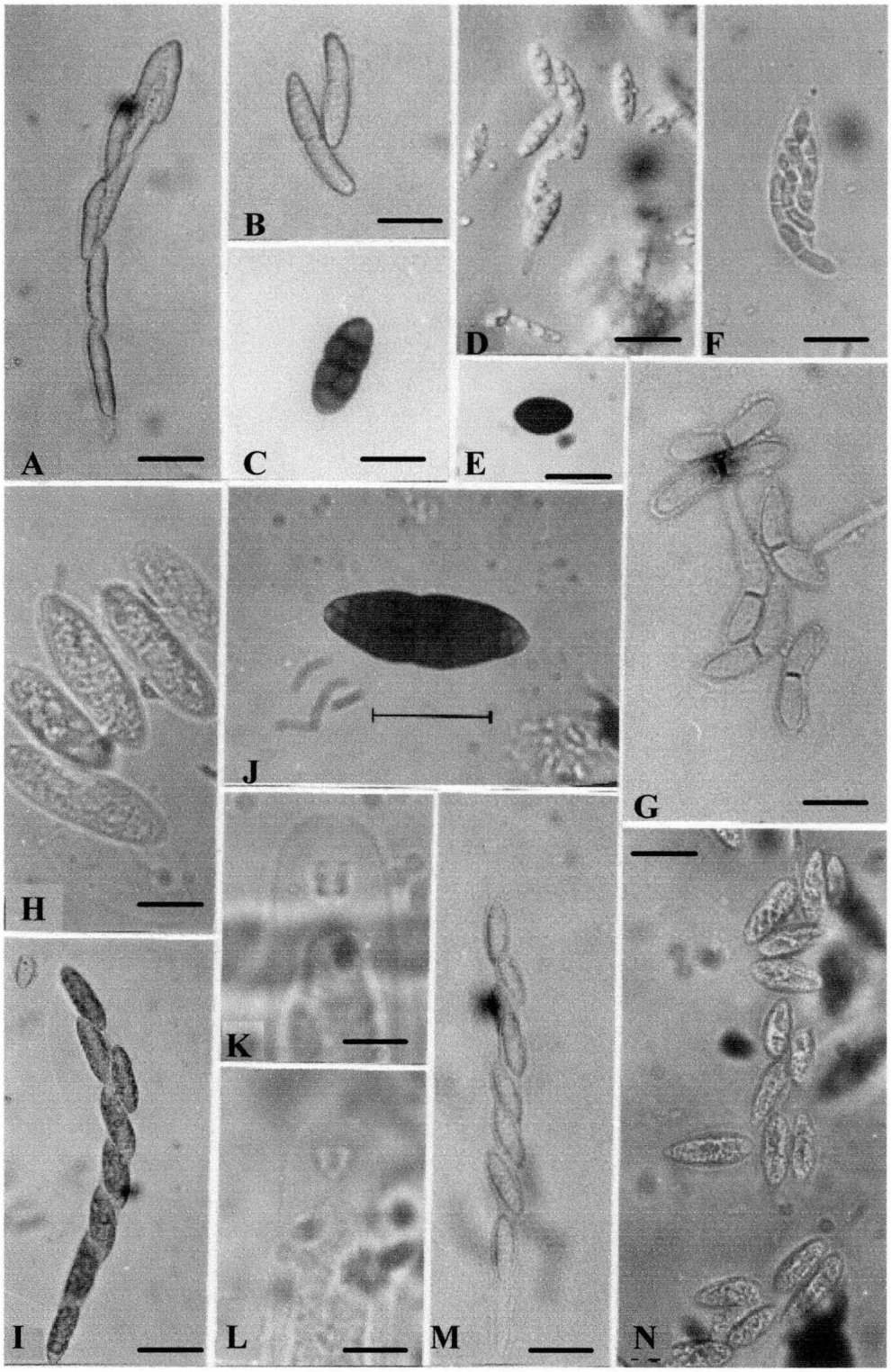
Figs.1.4–1: Ascomata

A, **B.** *Bertia antennaroidea:* ascomata on stem (W22a) and vertical section of ascoma with basal hyphal subiculum (holotype); **C.** *Coniochaeta pulveracea*: ascomata on stem; **D.** *Cryptodiaporthe curvata*: vertical section of ascomata showing peridium (W20); **E, F.** *Diaporthe fusispora*: ascomata on stem of *E. globulus* (holotype) and on stem of *E. nitens* (W22d); **G.** *Eutypa spinosa*: ascomata on stem (WC56); **H.** *Fenestella media*: Ascomata immersed in bark (VPRI 20830); **I.** *Karstenula ceanothi*: vertical section of ascoma (WC66a); **J.** *Phomatospora macrospora*: ascomata immersed in bark (W11b)



Bar = 5 μm **K**; 10 μm for **I** & **O**; 20 μm for **A**, **C**–**H**, **J**, **N** & **P**;
25 μm for **M**; 30 μm for **B** and 40 μm for **L**

Figs. 1.4–2: **A, B.** *Bertia antennaroidea* (asci and ascospores); **C.** *Karstenula ceanothi* (ascospores); **D.** *Fenestella media* (ascospore); **E, F.** *Diaporthe fusispora* (asci and ascospores); **G, H.** *Cryptodiaporthe curvata* (asci and ascospores); **I.** *Coniochaeta pulveracea* (ascospore); **J, K.** *Eutypa spinosa* (asci and ascospores); **L, M.** *Phomatospora macrospora* (ascospores and ascus); **N–P.** *Wuestneia campanulata* (ascospores, ascus apex and ascus)



Bar = 10 μm for **C–E** & **K–L**; 15 μm for **A**, **B**, **G** & **H**;
17.5 μm **M** & **N**; 20 μm for **F** & **J**

Figs.1.4–3: **A, B.** *Bertia antennaroidea* (asci and ascospores) (holotype); **C.** *Karstenula ceanothi* (ascospores); **D.** *Diaporthe fusispora* (asci and ascospores) (holotype); **E.** *Coniochaeta pulveracea* (ascospore); **F, G.** *Cryptodiaporthe curvata* (asci and ascospores) (holotype); **H, I.** *Phomatospora macrospora* (ascospores and ascus) (holotype); **J.** *Fenestella media* (ascospore); **K–N.** *Wuestneia campanulata* (ascus apex, ascus and ascospores) (holotype)

Phomatospora macrospora differs from other species in the genus by its large ascospores, hence the specific epithet. Species of *Phomatospora* have ascospores generally less than 25 μm long, while the present fungus on eucalypts has ascospores measuring 30–47.5 x 8.8–12.5 μm. Only one other species, *P. punctulata* Hino & Katumoto has comparably sized ascospores which measure 23–35 x 6.5–8 (mean 28.9 x 7.2) μm but they are contained in shorter asci (*fide* Kobayashi 1970). The woody habit of the perithecia and the relatively large, one-celled, cylindrical and nonpigmented ascospores of *P. macrospora* may suggest affinities with *Cryptospora* Tulasne. However, species of *Cryptospora* have perithecia embedded in stromatic tissue in circular groups with long ostiolar necks collectively erumpent, shorter asci with deliquescent bases, and the ascospores are subcylindrical, curved and normally longer than 50 μm (Müller and von Arx 1973; Dennis 1981).

12. *Therrya eucalypti* Z.Q. Yuan (Figs.1.4–4)

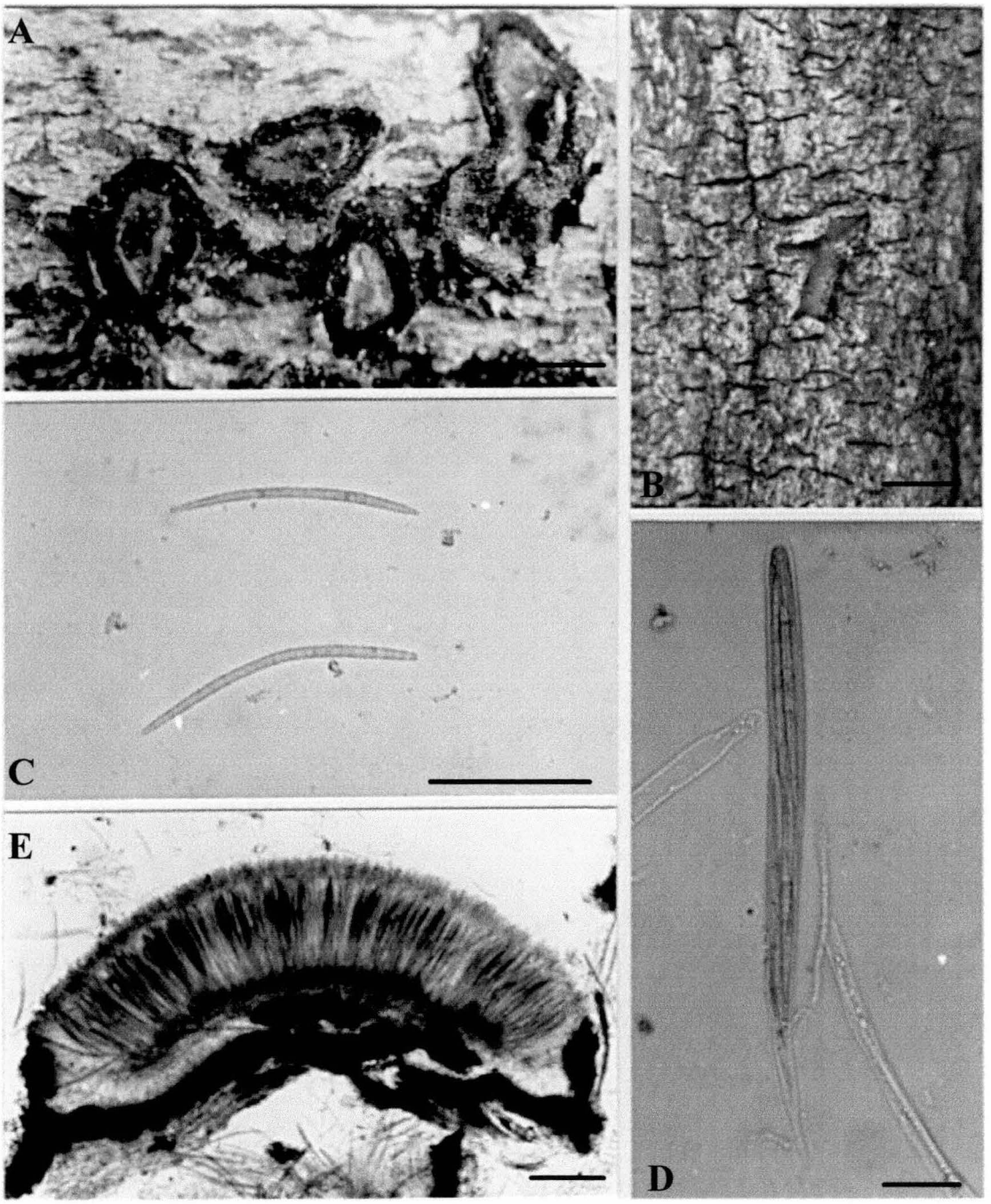
in Yuan and Mohammed, Mycotaxon 64: 174 (1997)

Ascostromata immersed to erumpent, sessile, solitary to gregarious in 2–5, round to elongate or irregular, 1–1.3 x 1.2–1.8 mm, 0.5–0.7 mm high; black; excipulum black, dehiscing by a single longitudinal split or splitting into unequal radiating fissures to expose the orange-colored hymenium; subhymenium hyaline, plectenchymatous; medullary excipulum 50–200 μm high. Asci 150–195 x 8–10 (mean 182 x 8.5) μm, unitunicate, cylindrical, rounded or blunt at apex, slightly thickened, without a I^+ blue apical ring, 8-spored, occasionally 6-spored. Ascospores 62–88 x 2–3.0 (mean 78 x 2.6) μm, hyaline, fusiform, straight or curved, 2–5 septate, in two fascicles in the ascus. Paraphyses filiform, slightly swollen at the apex and branched, agglutinated above the asci to form a epithecium.

On dead branches of *Eucalyptus regnans*.

Specimens examined: Tasmania: Florentine Valley, Westfield, on *Eucalyptus regnans*, 13 July 1995, *Z.Q. Yuan* 42 (VPRI 21091, Holotype); same location and host, 13 July 1995, *Z.Q. Yuan* 49; same location and host, 20 July 1995, *Z.Q. Yuan* 54; same location and host, 20 July 1995, *Z.Q. Yuan* 58.

Figs. 1.4-4: ***Therrya eucalypti***



Bar = 0.75 mm for **A**; 1.25 mm for **B**; = 40 µm for **C**; 22.5 µm **D**; 150 µm for **E**

A. Ascostromata (fully opened);
B. Ascostroma (half opened);
C. Ascospores with two and four septa;
D. A single ascus containing eight spores;
E. Longitudinal section of an ascostroma

Sections in C–E were stained in lactophenol cotton blue

Table 1.4–1. Morphological comparison of all the known species of the genus *Therrya* with *T. eucalypti*[a]

	T. pini	*T. fuckelii*	*T. piceae*	*T. pseudotsugae*	*T. tsugae*	*T. eucalypti*
Ascostroma	Scattered or clustered, oval, 1.5-3 mm wide; opening by longitudinal fissures or irregular lobes	Scattered or clustered, 3 mm diam.; opening irregularly by lobes or splitting longitudinally	Round or discoid, 0.6-1.1 mm diam., 0.6 mm high; opening by irregular radiating fissures	Round to undulating, 0.5-0.7 mm diam., 0.4 mm high; opening by unequal radiating fissures	Hysteriiform to irregular, 0.6 x 0.3 mm, 0.3 mm high; opening by a single longitudinal split	Round to irregular, 1-1.3 x 1.2-1.8 mm, 0.5-0.7 mm high; opening by a single longitudinal split or unequal fissures
Epithecium	Dark brown to black	Dark brown to black	Brown	Pale yellow	—	Orange
Ascus	Clavate, 90-135 x 12-16 µm; 8-spored	Clavate to cylindrical, 105-157 x 10-17 µm; 4-spored	Clavate, apex thickened, 80-150 x 10-12 µm; 8-spored	Clavate, apex thickened, 165-185 x 8-10 µm; 8-spored	Clavate, apex flattened, thickened, 150 x 12 µm; 8-spored	Cylindrical, apex slightly thickened, 150-195 x 8-10 µm; 6-8-spored
Ascospore	Needle-shaped, no appendages, 3-7-septate, 50-80 x 2.5-4.5 µm	Fusiform, with an apical and basal thread-like appendage, 7-11-septate, 64.5-110 x 3-5.5µm	Broadly fusiform to clavate, straight, 3-septate, 30-38 x 4-4.5 µm	Fusiform, straight, 3-6-septate, 48-72 x 2 µm	Fusiform, curved, pointed at both ends, 2-4-septate, 45-85 x 2 µm	Fusiform, curved, pointed at both ends, 2-5-septate, 62-88 x 2-3 µm
Paraphysis	Apex swollen, up to 6 µm	Apex swollen, up to 6 µm	Filiform, broadened at apex, branched	Filiform, slightly swollen at apex, branched	Filiform	Filiform, slightly swollen at apex, branched
Host plant	*Pinus* spp.	*Pinus* spp.	*Picea glauca*	*Pseudotsuga menziesii*	*Tsuga heterophylla*	*Eucalyptus regnans*
Distribution	Europe	Europe, N America	Canada	Canada	Canada	Australia

[a]Data for *T. pini* and *T. fuckelii* are from Reid & Cain (1961), for *T. piceae*, *T. pseudotsugae* and *T. tsugae* from Funk (1980)

The genus *Therrya* was originally established by Saccardo and Penzig (*Michelia* **2**, 604, 1882) for the type *T. pini* (Alb. & Schw.) von Höhn. (as *T. gallica* Sacc. & Penz.). Reid and Cain (1961) simplified the concept of the genus and accepted two species, *T. pini* and *T. fuckelii* (Rehm) Kujala. Funk (1980) described three new species which were parasitic on *Picea*, *Pseudotsuga* and *Tsuga* from North America. Since then, no more species have been added to this genus (Hawksworth *et al.* 1995).

Therrya was originally included in the family Phacidiaceae based on the characteristic that it lacked a mucilaginous sheath surrounding the fusoid ascospores (Korf 1973). Subsequently, Phacidiaceae was restricted by Di Cosmo *et al.* (1984) to accommodate those genera which have asci with an I^+ apical ring and hyaline, aseptate ascospores without a sheath. *Therrya* now belongs to Rhytismataceae (Hawksworth *et al.* 1995). The fungus reported here has been included in the genus *Therrya* based on the presence of: immersed, sessile ascostromata; I^- asci; and ascospores that are hyaline, fusiform, multiseptate and lack a mucilaginous sheath.

The main characteristics of all the six *Therrya* species are compared in Table 1.4-1.

Therrya eucalypti can be readily distinguished from other known species of the genus *Therrya* by its typically cylindrical asci and long, fusiform, septate ascospores which are arranged in two fascicles in the ascus. Of the known species of *Therrya*, *T. eucalypti* is closer to *T. tsugae* Funk in shape, size and septum number of the ascospores, but differs from the latter in shape and size of the asci and ascostromata (Table 1.4–1). After maturity, the ascostromata of *T. eucalypti* remain open to expose an orange-colored hymenium. This feature is similar to that observed in *T. pseudotsugae* Funk. In addition, all species previously reported were found on conifers in the northern hemisphere. This is the first record of a *Therrya* species on hardwood in the southern hemisphere.

13. *Valsa ceratosperma* (Tode) Mair

Publications de l'Indtitut Botanic Barcelona 3 (4): 20 (1937)

Syn.: *Shpaeria ceratosperma* Tode, Fungi Mecklenburgenses Selecti 2: 53 (1791)

Diatrype ceratsperma (Tode : Fr.) Fr.

Stroma immersed in branches, black erumpent, 1–2.5 mm in diam. Perithecia clustered in stroma, subglobose, with long necks converging through stroma, 200–500 μm in diam, black. Asci unitunicate, ellipsoid to clavate, with a nonamyloid refractive apical ring, 25–30 x 3.5–5.5 μm, 8-spored. Ascospores hyaline, 1-celled, allantoid, 7–9 x 1.5–2.0 μm.

On dead and dying branches of eucalypts.

Specimen examined: Tasmania: Barcoo Rd, Montagu, on *E. nitida*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC34; Ben Ridge, St. Helens, on *E. amygdalina*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET24; Black Jack's Hill, Little Swanport, on *E. amygdalina*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET6; Blue Gum Rd, Ridgley, on *Eucalyptus nitens*, 1 Aug. 1995, *Z.Q. Yuan* 69a; same location and date, on *E. globulus*, *Z.Q. Yuan* 74; Diddleum Plains, on *E. regnans*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET26; Goulds, Dover, *E. globulus*, 11 Aug. 1995, *Z.Q. Yuan* 81; Hastings, on *E. nitens*, Aug. 1995, *T. Wardlaw* 85; Henty Bridge, Strahan, on *E. regnans*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC11, WC17; Lime Bay, Tasman Peninsula, on *E. pulchella*, 4 July 1995, *Z.Q. Yuan* 29; Marlborough Highway, Miena, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET45; Mara Creek, Branxholm on *E. regnans*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET30; Mitchells Creek, Buckland, on *E. globulus*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET2; Navarre Plains, Derwent, on *E. obliqua*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC4b; Seed orchard, North Forest Products, Ridgley, on *E. delegatensis*, 1 Aug. 1995, *Z.Q. Yuan* 71; Tayatea Rd, Togari, on *E. nitida*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC48a; Trafalgar Flant, St. Helens, on *E. viminalis*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET17c; Ulverstone, on *Eucalyptus* sp., 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC59; Upper Dromedary, on *E. obliqua*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET59a; Woolnorth, on *E. nitens*, 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W4, W7, W9; same location, on *E. globulus*, 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W5, W8.

A *Valsa* species, often associated with *Cytospora eucalypticola* was frequently encountered during surveys. This fungus is close in morphology to *V. ceratosperma* which has been identified as the teleomorph of *C. eucalypticola* in south-east Australia (Old *et al.* 1991). It has not been previously reported in Tasmania.

14. *Wuestneia epispora* Z.Q. Yuan (Figs.1.4–5, 6, 7)

in Yuan and Mohammed, Mycological Research 101: 195 (1997)

Ascomata caulicolous, perithecial, single or aggregated in groups of 2–7, immersed, with perithecial necks emergent, ectostromatic disc furfuraceous brown to yellow-brown. Perithecia dark brown to black, spherical or sub-spherical, 200–450 µm diam. Peridium 15–20 µm wide, composed of dark brown, pseudoparenchymatous cells, 4–7 µm diam. Perithecial neck cylindrical to subcylindrical, up to 65 µm wide at the base and 100 µm long. Asci 100–188 x 13–33 µm (mean = 128 x 22 µm), unitunicate, clavate, short-stalked, mostly with obtuse to almost truncate and strongly thickened apex, but no apical apparatus seen and not blueing in iodine, 8-spored (rarely 4–, 6–, or 7–spored). Ascospores 20–35 x 9–15 µm (mean = 28 x 12 µm), obliquely uniseriate or biseriate, hyaline, fusoid to ellipsoidal, obtuse at each end, aseptate, thick-walled, smooth, granular, surrounded by a thick (2–8 µm), persistent gelatinous sheath.

On stems of *Eucalyptus* spp.

Specimens examined. Tasmania: Goulds, on *Eucalyptus nitens*, 11 Aug. 1995, *Z.Q. Yuan* 79, Holotype (VPRI 20786); Burnie, on *E. nitens*, 1 Aug. 1995, *Z.Q. Yuan* 70(VPRI 20787); same locality, on *E. delegatensis* R. Baker, 1 Aug. 1995, *Z.Q. Yuan* 72 (VPRI 20788); Hastings, on *E. nitens*, Aug. 1995, *T. Wardlaw* 83 (VPRI 20789); Diddleum Plains, on *E. nitens*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET27 (VPRI 20823); Upper Dromedary, on *E. obliqua* L'Hér., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET59 (VPRI 20824).

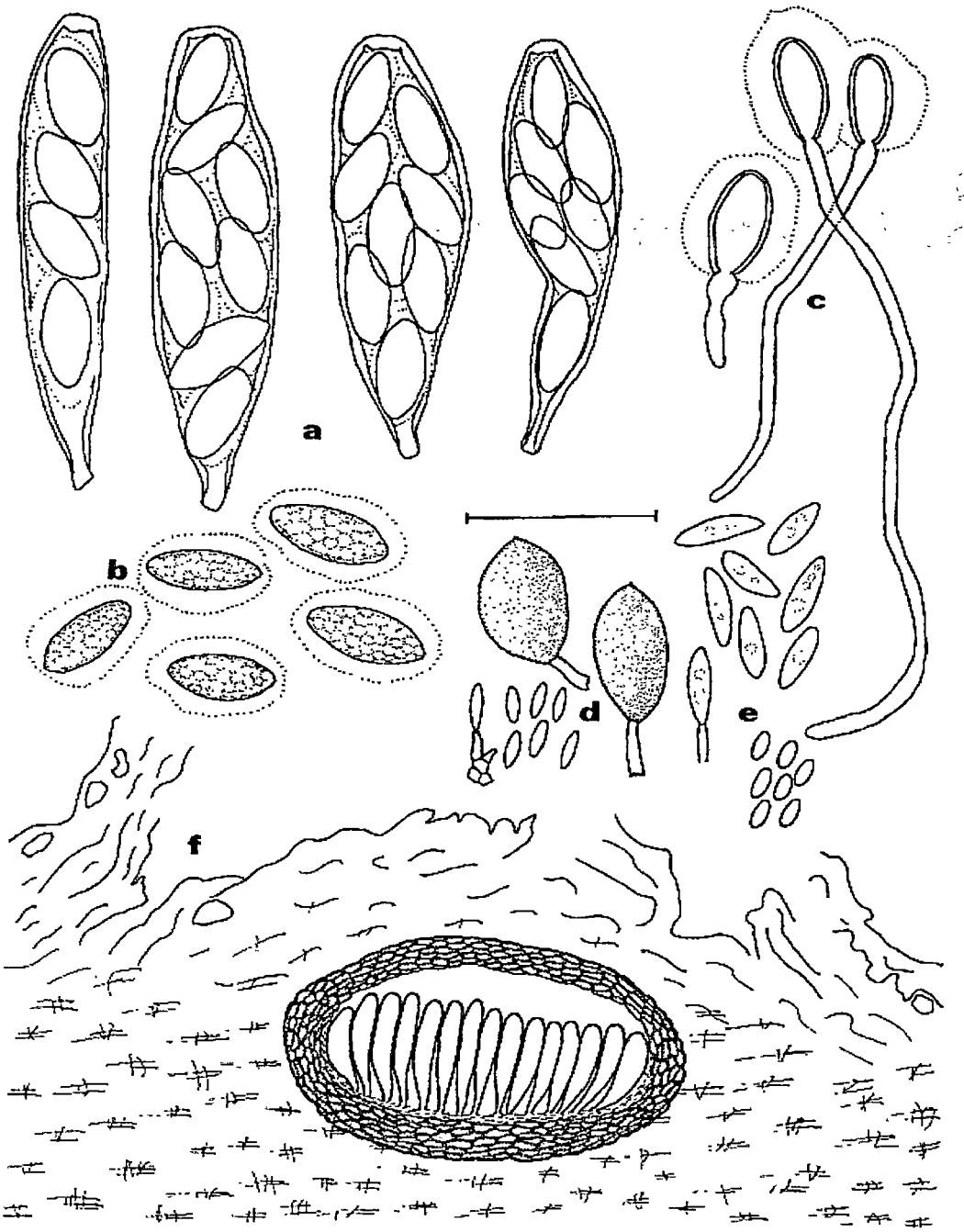
The genus *Wuestneia* was introduced by Auerswald (in Fuckel, Fungi Rhenani No. 587, 1863). For a long time, the genus was regarded as a synonym of *Cryptosporella* Sacc. (Hawksworth *et al.* 1983). It was not until 1988 that Ananthapadmanaban adopted *Wuestneia* as the correct generic name for fungi previously assigned to *Cryptosporella* (Ananthapadmanaban 1988). Reid and Booth (1989) gave an account of the genus and reduced *Cryptosporella* to synonymy with *Winterella* O. Kuntze. According to Reid & Booth, species of *Wuestneia* are characterised by orange to yellow-brown ectostromatic and subhyaline to pale brown entostromatic layers of the stroma, circinately-arranged perithecia with beaks erumpent through the ectostromatic disc, and by one-celled, hyaline, ellipsoidal to inaequilateral ascospores which may

have a mucilaginous sheath. Anamorphs of *Wuestneia* form two groups: (*i*) with ellipsoidal to inaequilateral, hyaline, aseptate, thick-walled conidia and (*ii*) with doliiform, pigmented and appendaged conidia which are referable to *Harknessia* Cooke (Reid and Booth 1989; Nag Raj and DiCosmo 1981).

Ten species and one variety were accepted in the genus by Reid and Booth (1989). Since then two more species have been proposed; one from South Africa on eucalypts (Crous *et al.* 1993) and one (as *Cryptosporella*) from Australia on acacias (Sutton and Pascoe 1989).

Two different anamorphs, both of which produce microconidial synanamorphs, were associated with *W. epispora*. One unnamed hyaline non-appendaged anamorph was derived from the holotype of *W. epispora* (VPRI 20786) and stored as dried agar sheet (VPRI 20790) (Figs.1.4–5*d*; 1.4–7*c,d*). This anamorph is characterized by having eustromatic conidiomata, conidiogenous cells proliferating percurrently, conidia fusoid to ellipsoidal, straight or inaequilateral, hyaline, 15.5–22.5 x 4.5–6.0 (mean = 19.6 x 5.1) µm, collaret absent, with 1–3 small guttulets and by its microconidia ellipsoidal, hyaline, 6.5–7.5 x 2.5–4.5 µm. The other is a pigmented, appendaged *Harknessia* sp, possibly *H. eucalypti* which is detailed in section 1.4.2.

Two species of *Wuestneia* from section *Wuestneia*, *W. acericola* J. Reid & C. Booth and *W. xanthostroma* (Mont.) J. Reid & C. Booth have been recorded as having anamorphs with hyaline, non-appendaged conidia. Only *W. acericola* has two types of hyaline conidia (Reid and Booth 1989). Although *W. epispora* is similar to *W. acericola* in that two types of hyaline conidia are present, the shape of both conidium types is different to those of *W. acericola*. The microconidia of *W. acericola* are rod-shaped, to curved and tapered towards the ends and the macroconidia are spatulate, whereas the microconidia of *W. epispora* are typically ellipsoidal (6.5–7.5 x 2.5–4.5 µm) and the macroconidia fusoid. The latter are also much narrower than those of *W. acericola* (6–8.5 µm wide, *fide* Reid and Booth 1989).

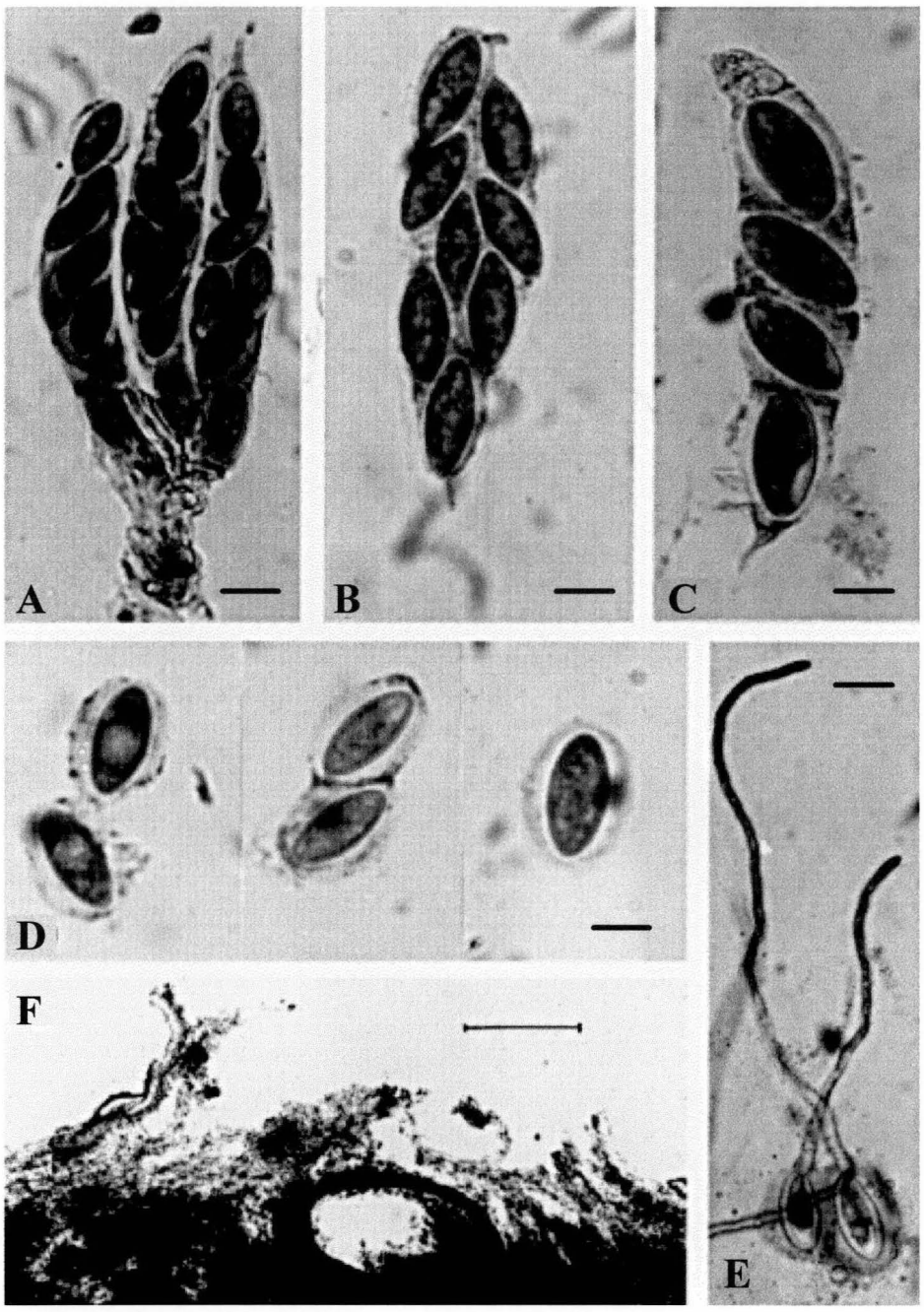


Standard line = 45 µm for **A–C**; 35 µm for **D**; 40 µm for **E**; 200 µm for **F**

Fig. 1.4-5: ***Wuestneia epispora***

A. Asci (note 4– & 7–spored asci); **B.** Ascospores with thick gelatinous sheath and granular contents; **C.** Germinating ascospores; **D.** *Harknessia* cf. *eucalypti*, macro- and microconidia; **E.** Unnamed hyaline, non-appendaged-anamorph, macro- and microconidia; **F.** Ascoma in vertical section

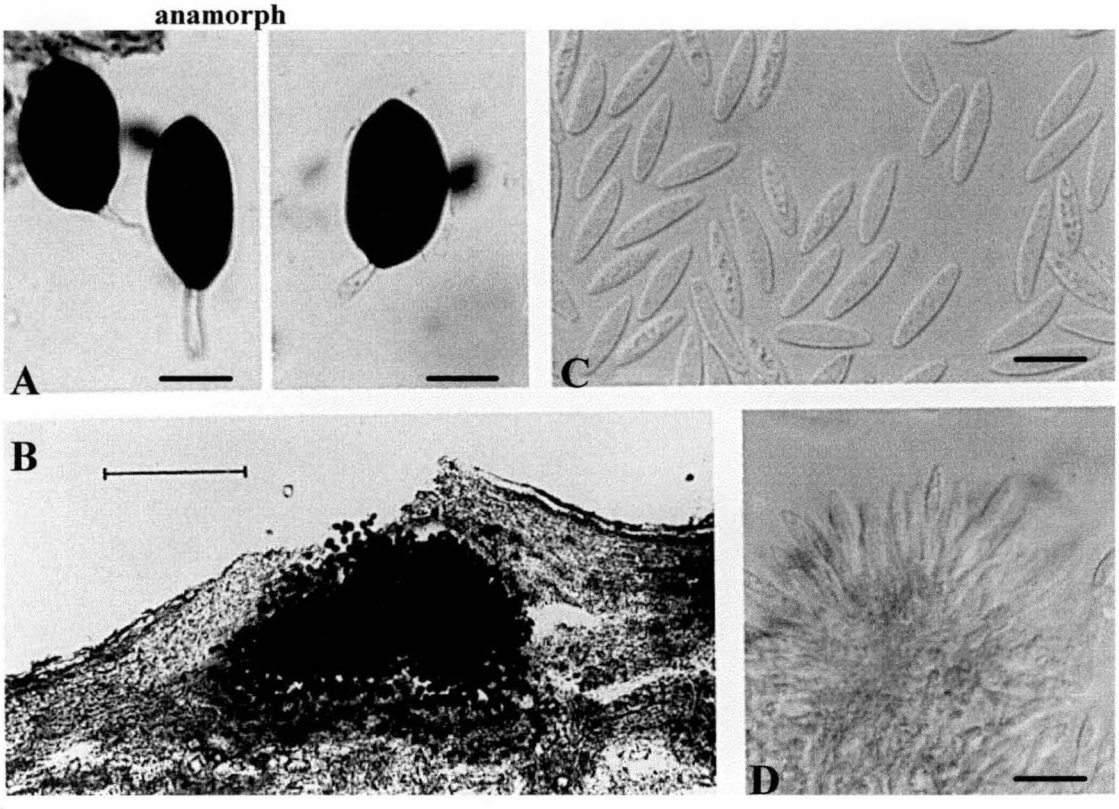
Fig. 1.4–6: ***Wuestneia epispora***



Standard line = 12.5 μm for **B–D**; 15 μm for **A**, **E**; 200 μm for **F**

A-C. Asci;
D. Ascospores with thick gelatinous sheaths;
E. Germinating ascospores;
F. Ascoma in vertical section

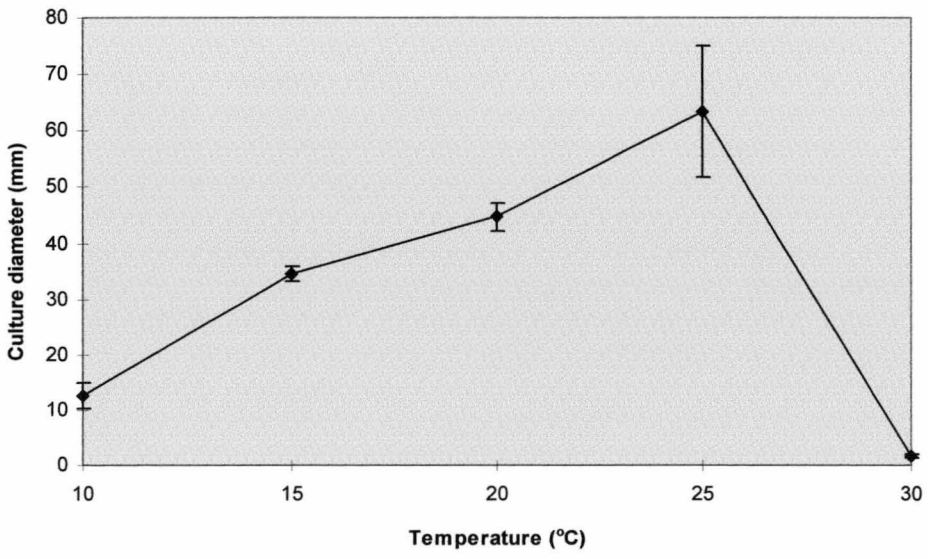
Fig. 1.4-7: *Harknessia* cf. *eucalypti* and the unnamed hyaline, non appendaged-anamorph



Standard line = 10 μm for **A**; 14 μm for **C**, **D**; 250 μm for **B**

A. Conidia of *H.* cf. *eucalypti*; **B.** A vertical section of conidioma of *H.* cf. *eucalypti*; **C.** Conidia of the unnamed anamorph; **D.** Developing conidia and conidiophores of the unnamed anamorph

Fig. 1.4-8: Mean diameter growth (± standard error of the mean) of three replicates of *Wuestneia epispora* on MEA after 25 days incubation at various temperatures



Species of *Wuestneia* have been recorded either with pigmented or non-pigmented anamorphs (Reid and Booth 1989). This is the first time that both anamorphs have been found in one species.

Of the previously known species assigned to the genera *Wuestneia* and *Cryptosporella* with *Harknessia* anamorphs, only two species, *W. beltsvillensis* (Petrak) J. Reid & C. Booth and *Wuestneia* sp (Reid and Booth 1989) have ascospores with gelatinous sheaths. However both of these species have much smaller asci and ascospores than those of *W. epispora*. Also the different *Harknessia* anamorphs have conidia with both apical and basal appendages (Barr 1978; Reid and Booth 1989). In terms of *Harknessia* anamorphs only *W. eucalyptorum* Crous, Wingfield & Nag Raj on eucalypts and *Cryptosporella karvarrae* from Australia are comparable. *Wuestneia epispora*, however, differs from these others in a number of features such as habit, ascus type and size, ascospore shape, size and gelatinous sheath etc. (Table 1.4–2).

Wuestneia epispora is well distinguished from the known species in the genus by its large, 4- to 8- spored asci and ascospores with firm, thick gelatinous sheaths, as well as its two types of synanamorphs. The fungus has the longest and widest asci in the known species of *Wuestneia*. Most of the species have asci ranging from 50–130 µm long and 8–20 µm wide (Reid and Booth 1989), while the asci in *W. epispora* measure 100–188 x 13–33 µm.

Colony growth rate on MEA was studied from 10°–30 °C (Fig. 1.4–8). The result shows that the fungus grows slowly at different temperatures reaching a maximum mean diameter of 63.3 mm after 25 days incubation at its optimum temperature of 25 °C.

Colonies are straw-coloured, fairly compact, with orange to yellow-brown reverse, and do not change colour when exposed to light. Colonies form small sectoring growth pattern with dense concentric rings. After one week incubation under light on MEA and two weeks on wheat-rice bran, dark brown conidiomata extruding conidia

were produced at the margin of the agar and at the bottom of flasks. Ascomata and ascospores were also produced on wheat/rice bran.

As the generic name *Cryptosporella* has been reduced to synonymy with *Winterella* (Reid and Booth 1988), *Cryptosporella karvarrae* B. Sutton & I. Pascoe described on acacias from Australia (Sutton and Pascoe 1989) has been transferred into *Wuestneia* by Yuan and Mohammed (1997b).

Table 1.4–2: Comparison of *Wuestneia epispora* and two other species of *Wuestneia* with *Harknessia* anamorphs with conidia with basal appendages only

	W. epispora	*W. eucalyptorum**	*Cryptosporella karvarrae***
Host	*Eucalyptus* spp.	*Eucalyptus* spp.	*Acacia glaucoptera*
Habit	on stems	on leaves	on stems and leaves
Ascus type	4-, 6-, 8-spored	8-spored	8-spored
Ascus size	100-188 x 13-33 µm	70-110 x 13-20 µm	75-90 x 17-20 µm
Ascospore shape	fusoid to ellipsoidal	ellipsoidal	ellipsoidal
Ascospore size	20-35 x 9-15 µm	13-28 x 8-13 µm	23-27 x 9-11 µm
Ascospore content	granular	guttulate	eguttulate
Gelatinous sheath	with a thick gel sheath	no gel sheath	no gel sheath
Anamorph	***Harknessia* cf. *eucalypti***	***H. eucalyptorum***	***H. karvarrae***
Conidial shape	narrowly ellipsoidal, ovoid, or broadly ventricose,	broadly ventricose	ellipsoidal, ventricose
Conidial size	18-35 x 12-16 µm	16-29 x 9-14 µm	17-18 x 12-12.5 µm
Appendage length	5-20 µm	3-16 µm	3-7 µm
Microconidium	fusoid, 5-10 x 2-4 µm	ellipsoidal to fusoid 4.5-9 x 2-3.5 µm	not found
Synanamorph	with hyaline, non appendaged conidia and microconidia		

* Based on Crous *et al.*, 1993; ** Based on Sutton and Pascoe, 1989

15. ***Wuestneia campanulata*** Z.Q. Yuan (Figs.1.4–2n-p; 1.4–3k-n; 1.4–9c)
in Yuan and Mohammed, Mycotaxon 63: 20 (1997)

Ascomata perithecial, single or in groups of 2–5, immersed, with emergent perithecial necks, appearing on the host surface as prominent oval pustules, up to 1.5 mm diam, ectostromatic disc furfuraceous brown to yellow-brown. Perithecia dark brown, spherical or subspherical, 350–550 µm diam. Perithecial neck cylindrical to subcylindrical, up to 80 µm wide at base and 350 µm long. Peridium 15–20 µm wide, composed of several layers of dark brown pseudoparenchymatous cells, 4–8 µm diam. Asci 100–150 x 12.5–17.5 (mean = 132 x 15) µm, unitunicate, cylindrical to sub-cylindrical, rounded at apex, with a distinct, campanulate, apical apparatus; 3.5–5.0 µm wide, 4–5 µm high, not blueing in iodine, 8-spored (rarely 4-spored). Ascospores 17.5–27.5 x 6.5–10 (mean = 22 x 8) µm, obliquely or irregularly uniseriate, hyaline, elliptic-fusoid to obovoid with one end somewhat acute, aseptate, thick-walled, smooth, granular.

On stems of eucalypts.

Specimens examined: Tasmania: Pine Tree Rivulet on *Eucalyptus pauciflora*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET44a (VPRI 21081, holotype); Black Jack's Hill, Little Swanport, on *E. amygdalina*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET8b (VPRI 21082); Pine Tree Rivulet, on *Eucalyptus* sp, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET43a (VPRI 21083); Fingerpost, Waratah, on *E. nitens*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC27 (VPRI 21084); Welcome River, Montagu, on *E. ovata*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC37b (VPRI 21085); Saggers Hill on *E. nitens* (?),13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC64 (VPRI 21086); Woolnorth, on *E. globulus*, 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W10 (VPRI 21087).

Two species of the genus *Wuestneia*, *W. eucalyptorum* Crous, Wingfield & Nag Raj and *W. epispora* Z. Q. Yuan have been previously recorded on eucalypts (Crous *et al.* 1993; Yuan and Mohammed 1997b).

Wuestneia campanulata is morphologically different from *W. epispora*. The asci of *W. epispora* are clavate, large (100–188 x 13–33 µm), and asci do not have apical

apparatus. Asci of *W. campanulata* are much smaller (mean 132 x 15 μm), cylindrical to subcylindrical, with ascospores monostichously arranged, and characteristically with a distinct, campanulate, 'annellascé' apical apparatus (*sensu* Chadefaud 1942) (Figs.1.4–2*o*; 1.4–3*k-m*; 1.4–9*c*). The ascospores of *W. campanulata* are also smaller than those of *W. epispora* and elliptic-fusoid to obovoid, without any gelatinous sheath surrounding them. In addition, the perithecium diameter of *W. campanulata*, a main criterion used by Reid and Booth (1989) to separate species of *Wuestneia*, is larger (up to 550 μm and mostly 500 μm) than that of *W. epispora*. Apart from the above morphological differences, *W. epispora* appears commonly associated with lower crown death in plantation eucalypts, whilst *W. campanulata* was more often found in native forests. *W. eucalyptorum* differs from *W. campanulata* in its absence of ascal apical apparatus and shape of ellipsoidal ascospores, as well as the phyllodiicolous habit (Crous *et al.* 1993).

In the key to species of *Wuestneia* provided by Reid and Booth (1989) *W. campanulata* keys out to *W. beltsvillensis* (Petrak) J. Reid & C. Booth. However, the latter can be easily separated from *W. campanulata* by (*i*) smaller perithecia (200–425 μm diam.) that are always in groups (4–15) with thicker peridium (up to 40 μm); (*ii*) an ascal apical apparatus which is only visible when mounted in water (while that of *W. campanulata* is always visible in both water and other mounting media, eg. in glycerol); (*iii*) shorter but wider (70–140 x 12–22.5 μm) asci and (*iv*) slightly smaller ascospores which are more fusiform in shape with a thin mucilaginous sheath when mounted in water. In addition, *W. beltsvillensis* seems to be restricted to *Liquidambar* L., a genus of the Hamamelidaceae (Reid and Booth 1989).

The ascal apical apparatus observed in many species of *Wuestneia* has been described as two refractive bodies (Barr 1978; Ananthapadmanaban 1988; Reid and Booth 1989), but has not been previously observed in detail. Based on Parguey-Leduc *et al.* (1994), the apical apparatus in the asci of *W. campanulata* matches Chadefaud's "annellascé" type being composed of two parts: an upper ring and a lower ring as illustrated in Figs.1.4–2*o* and 1.4–3*k, l*. The upper ring is composed of several rings and is superimposed on a lower single bowl-shaped ring.

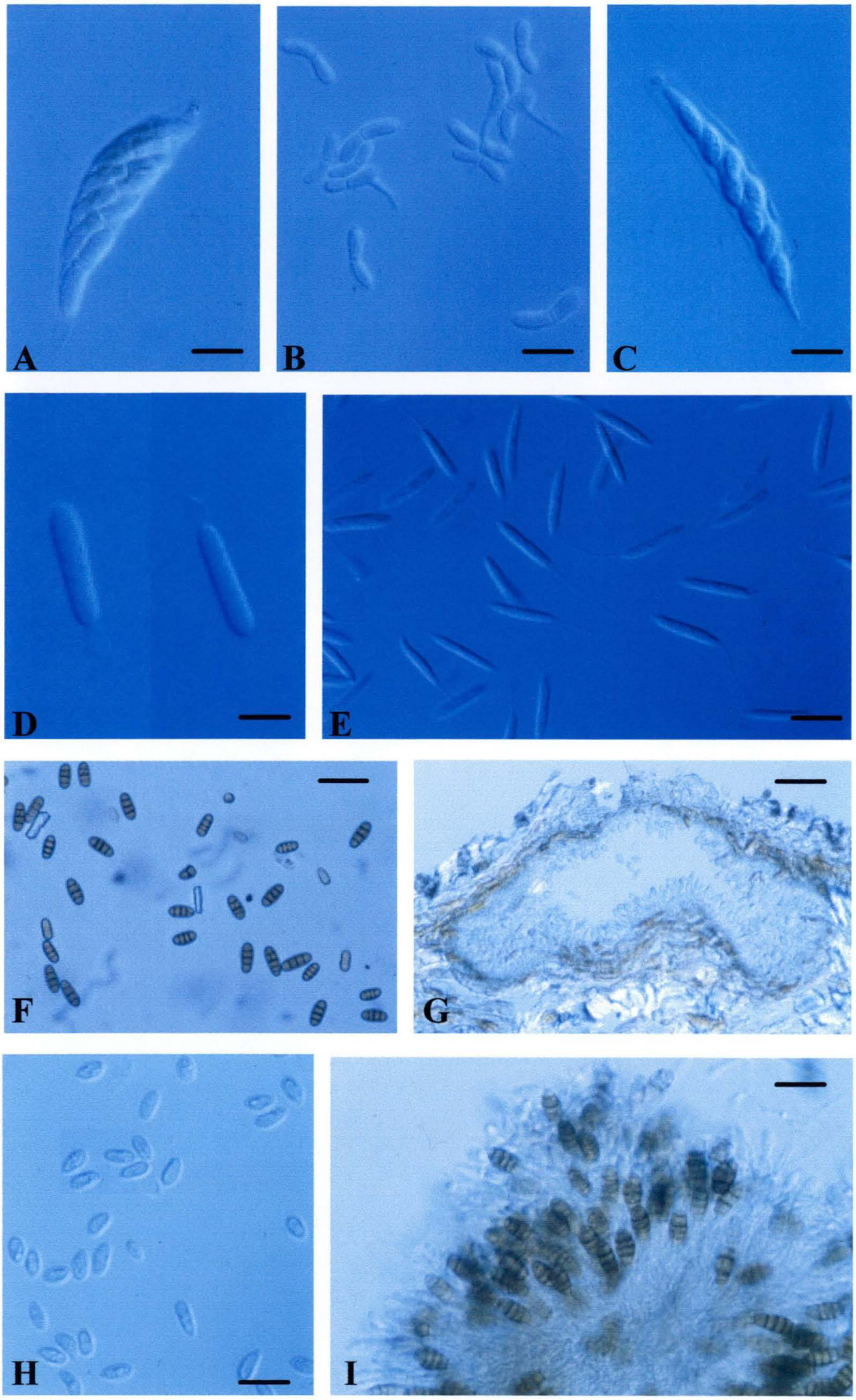
Fig. 1.4–9: Ascospores and conidia

A, B. Ascus and ascospores of *Cryptodiaporthe curvata*;
C. Ascus of *Wuestneia campanulata*;
D. Conidia of *Ceuthospora innumera*;
E. Conidia of *Neoplaconema cymbiforme*;
F. Conidia of *Camarosporium propinquum*;
G, H. Longitudinal section of conidioma and conidia of *Cryptosporiopsis* sp.;
I. Conidia and conidiophores of *Seiridium papillatum*

Fig. 1.4–10: Conidia and conidiomata immersed in stems

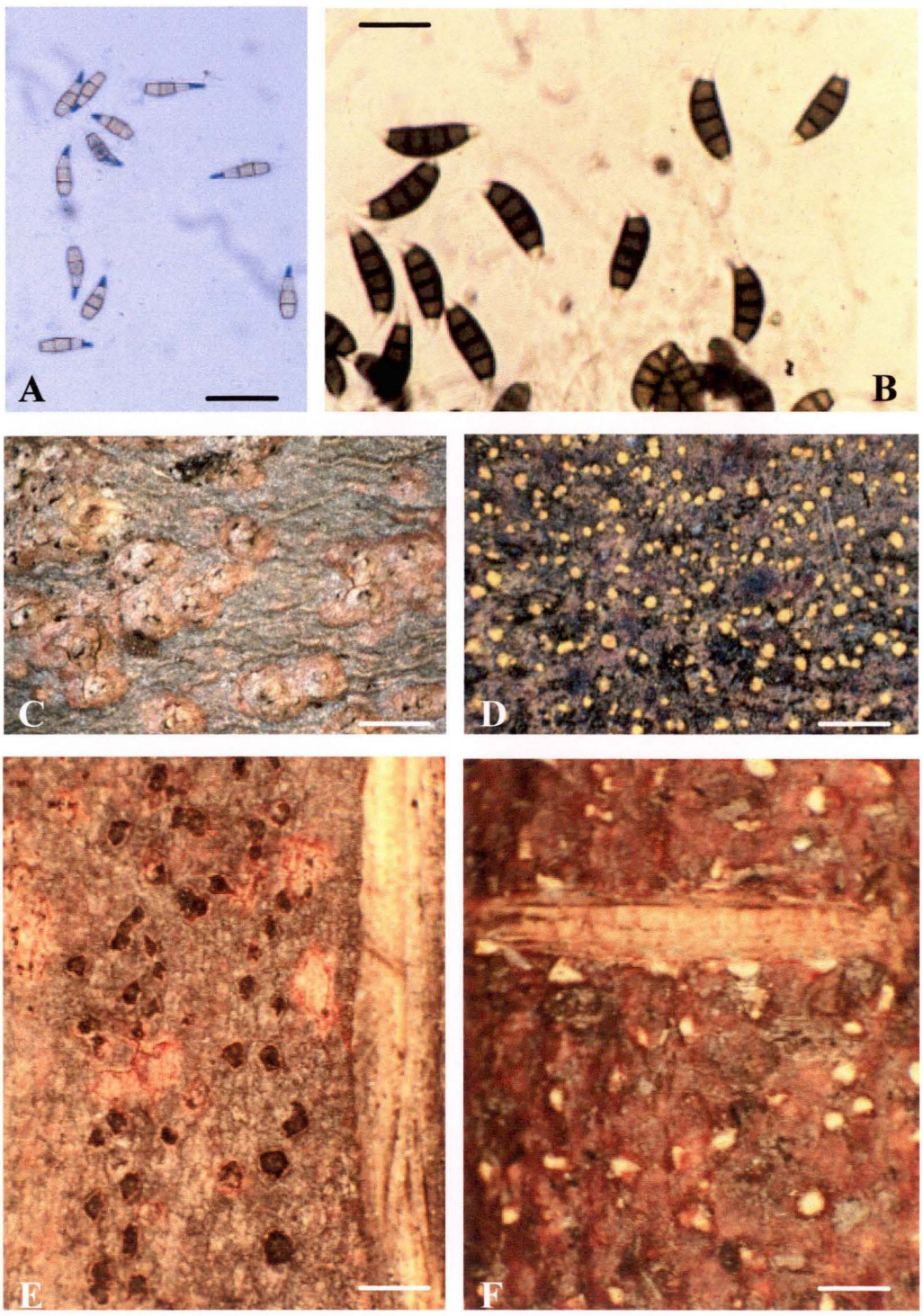
A. Conidia of *Pestalotiopsis neglecta* isolated from healthy bark of *Eucalyptus nitens*;
B. Conidia of *Seiridium eucalypti* on *E. regnans*;
C. Conidiomata of *Ciliosporella tuberculiformis* in wart-like swellings of *E. regnans* branch;
D. Conidiomata of *Endothia gyrosa* on *E. nitens*;
E. Conidiomata of *Zythiostroma* sp. on *E. obliqua* (WC58);
F. Microconidiomata of *Harknessia* cf. *eucalypti* (VPRI 20791)

Fig. 1.4–9: Ascospores and conidia



Bar = 7.5 µm for **D**; 10 µm for **E**; 12.5 µm for **A-C**; 30 µm for **H**, **I**; 40 µm for **F**; 60 µm for **G**

Fig. 1.4–10: Conidia and conidiomata immersed in stems



Bar = 20 µm for **A, B**; 1 mm for **C-F**

1.4.2 MITOSPORIC FUNGI

1. *Camarosporium propinquum* (Sacc.) Sacc. (Fig. 1.4–9f)

Syll. fung. 3: 464 (1844)

Syn.: *Hendersonia propinqua* Sacc.Michelia 1: 516 (1898).

Camrosporium populi Qud., Verh. K. Akad. Wet., ser. 2, 11: 541 (1904).

Conidiomata caulicolous, pycnidial, separate, immersed in branches, dark brown, subglobose, 400-550 µm wide, 250-300 µm high; wall 10-18 µm thick. Conidiogenous cells holoblastic, doliiform, 3-7 x 3-6 µm. Conidia 15-20 x 6.5-8.0 µm, ellipsoidal to elongate, with obtuse ends, light brown to brown, with 3 transverse septa, 1-2 longitudinal septa, not constricted at septa; wall smooth or slightly verruculose.

On dead branches of eucalypts.

Specimens examined: Tasmania: Seed orchard, North Forest Products, on *E. delegatensis* R. Baker, 1 Aug. 1995, *Z.Q. Yuan* 68; Jackeys Marsh, on *E. delegatensis,* 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET37b; Pine Tree Rivulet, on *E. pauciflora, Z.Q. Yuan & M. Hall* ET37.

Camarosporium eucalypti G. Winter has been the only other species found on eucalypts to be described under the genus *Camarosporium* Schulz. though it was later transferred to the genus *Dichomera* (Sutton 1975). The present fungus collected from several localities in Tasmania has typical characteristics of *Camarosporium* and its conidial morphology is very similar to that of *C. propinquum* (Sutton 1980). *Camarosporium propinquum* is common on hardwoods in the northern hemisphere. This is the first record of this fungus on eucalypts in the southern hemisphere.

2. *Ceuthospora innumera* Massee (Fig. 1.4–9d)

Bull. Misc. Inf. Roy. Grad. New: 102 (1899)

Conidiomata caulicolous, stromatic, pycnidioid, scattered, immersed with neck extending out of host bark, 4-6 per pustule, subglobose or ellipsoid, 110-180 µm wide, 200-250 µm high. Conidiophores slightly septate and branched at the base, 20-40 µm

long, invested in mucus. Conidia subcylindrical with a rounded base, slightly attenuated towards the apex, hyaline, smooth-walled, 15-22.5 x 2.5-3.8 μm, bearing a funnel-shaped, mucoid appendage at the apex.

On dead branches of *E. nitens*.

Specimens examined: Tasmania: Goulds, Hounville, on *E. nitens*, 11 Aug.. 1995, *Z.Q. Yuan* 78.

The type specimen of *C. innumera* was collected on dead leaves of *Eucalyptus* sp. in Tasmania (Nag Raj 1993). This species has not been found outside Australia, and to date, it has only been found on the leaves of eucalypts. This is the first record of the fungus on eucalypt stems.

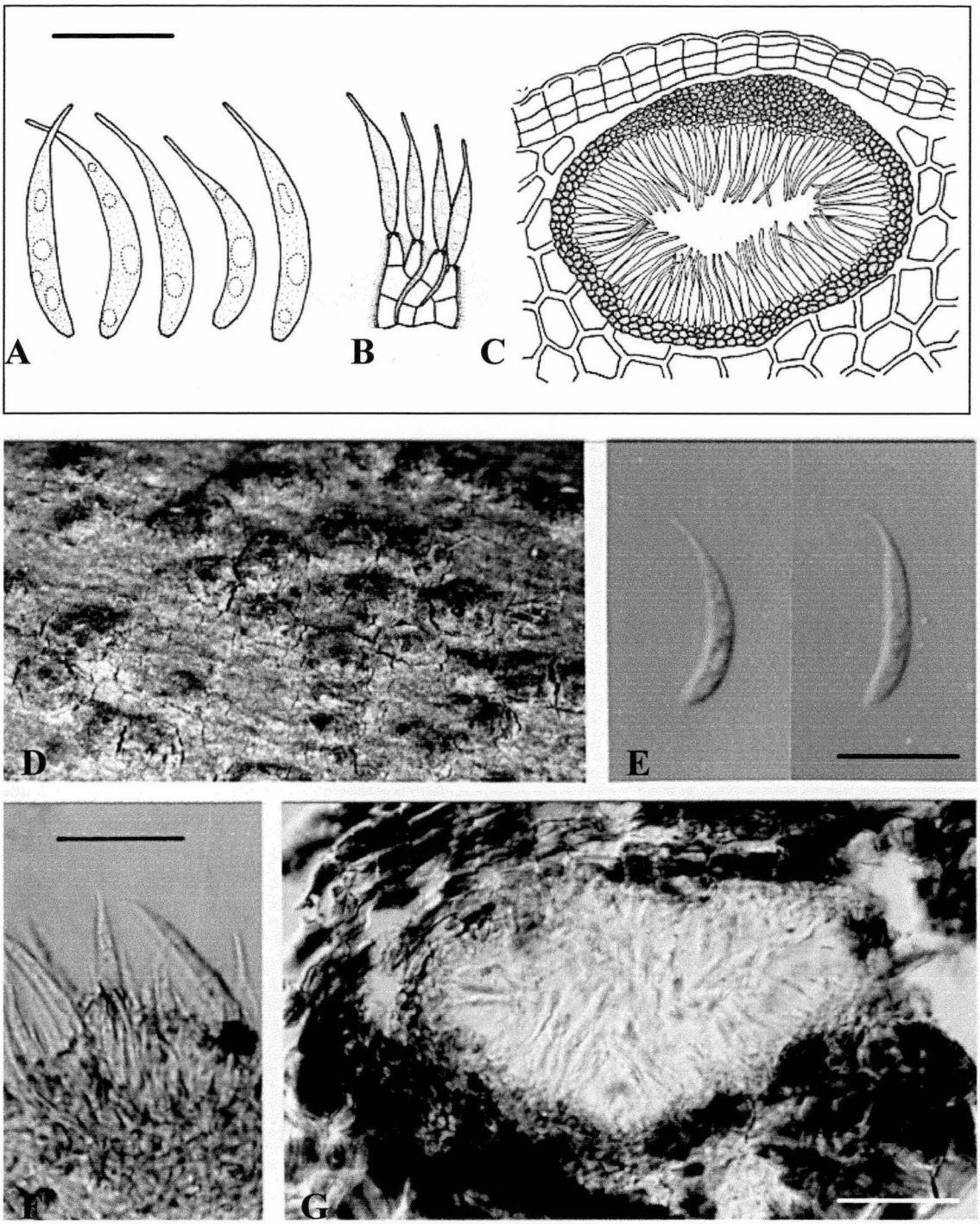
3. *Ciliosporella tuberculiformis* Z. Q. Yuan & C. Mohammed (Fig. 1.4–10c; 1.4–11)
Mycological Research 101: 1531 (1997)

Caulicolous. Conidiomata pycnidial, immersed, scattered or gregarious in groups at top of wart-like swellings of stems, globose to subglobose, small, 125-195 μm wide, 100-175 μm high, dehiscence by a split in the apical wall; wall 15-20 μm thick, up to 25-30 μm thick at top parts, composed of *textura angularis*, cells thick-walled, brown, becoming paler and thin walled toward the conidial hymenium. Conidiophores lining the cavity of the conidioma, hyaline, 1-3-septate, branched, smooth, up to 15 μm long, invested in mucus. Conidiogenous cells discrete, ampulliform to lageniform, hyaline, 5-7.5 x 2.5-3 μm. Conidia 30-45 x 3.8-5.0 (mean 38 x 4.8) μm, one-celled, hyaline, fusiform to naviculate, mostly curved, with a truncate base and an acute apex, smooth, guttulate and granular; apex attenuated into a rostrate, tubular, unbranched, cellular appendage (type A, *sensu* Nag Raj 1993), not separated from the conidium by a septum, 4.5-7.5 (mean 5) μm long; mean conidium length/width ratio = 7.9:1.

On dying branches of *Eucalyptus regnans*.

Specimens examined: Tasmania: Florentine Valley, Westfield, on *E. regnans*, 15 Feb. 1996, *Z.Q. Yuan* 93 (VPRI 20828), Holotype; same locality and host, 22 Sept. 1996, *Z.Q. Yuan* 296.

Fig. 1.4–11: ***Ciliosporella tuberculiformis***



Bar = 20 µm for **A** & **B**; 50 µm for **C**; 2 mm **D**; 25 µm for **E** and **F**; 40 µm for **G**

A. Conidia; **B.** Conidiophores; **C.** Vertical section of conidioma; **D.** conidiomata associated with wart-like swellings in necrotic lesions caused by stem tip dieback; **E.** conidia; **F.** Conidiophores; **G.** A vertical section of conidioma

The genus *Ciliosporella* was proposed by Petrak (*Annals mycologici* **25**: 217, 1927), based on the mitosporic type species *C. selenospora* Petrak. So far the genus has remained monotypic (Hawksworth *et al.* 1995). *Ciliosporella selenospora* was described on stems of *Trifolium alpestre* in Austria and is characterised by fusiform to naviculate, one-celled, hyaline conidia bearing an apical rostrate, tubular, unbranched appendage of type A (*sensu* Nag Raj 1993).

Ciliosporella tuberculiformis fits the concept for the genus *Ciliosporella* given by Nag Raj (1993), even though it is very different from *C. selenospora* in its host and geographical distribution.

Ciliosporella tuberculiformis differs from *C. selenospora* in that its conidiomata are always associated with wart-like swellings on dying branches of eucalypt and the conidiomatal wall is characteristically thicker at its apex. Conidia in *C. tuberculiformis* are curved in shape, filled with guttules and granules, whilst those of *C. selenospora* are straight, without contents, and much smaller (20-29 x 2-3 µm for *C. selenospora, fide* Nag Raj 1993).

Conidial morphology in *Ciliosporella tuberculiformis* is also similar to that of *Monochaetiella hyparrheniae* Castellani which has fusiform to naviculate, unicellular conidia bearing an apical appendage of type A (Nag Raj 1993). However, *M. hyparrheniae* has conidiomata of typical acervuli and much wider conidia with a mean conidium length/width ratio of 5.7:1.

Conidia of *C. tuberculiformis* were found associated with stem canker in the field and were even fruiting on the healthy area adjacent to the canker. However, isolations made from tissue exhibiting conidia only produced isolates of *Endothia gyrosa* (Schw.:Fr.) Fr. and *Cytospora eucalypticola* van der Westhuizen (Yuan, unpublished data).

4. *Cryptosporiopsis* sp. (Fig. 1.4–9g,h; 1.4–12)

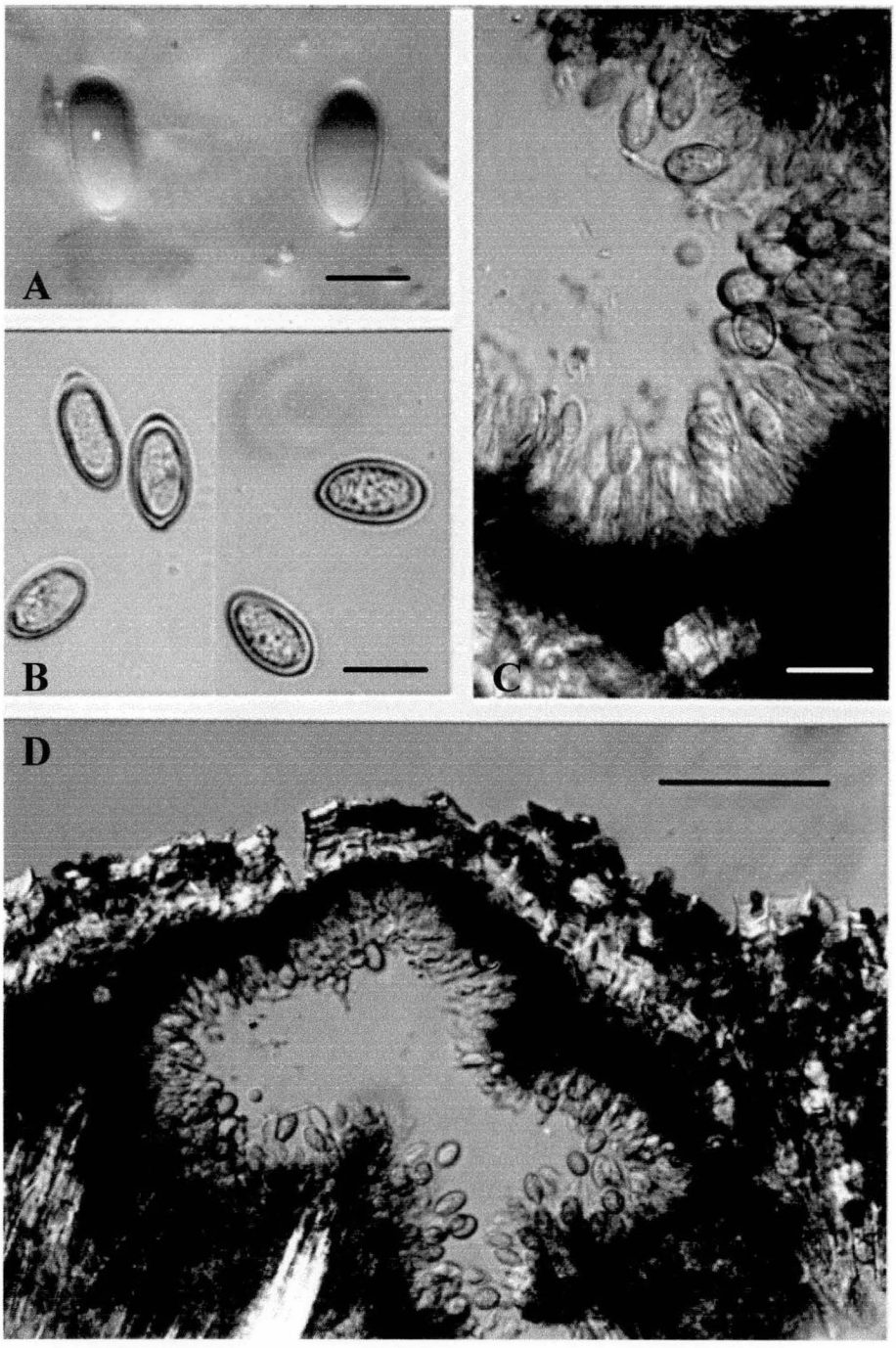
Conidiomata caulicolous, acervular to stromatic, immersed, scattered, up to 600 µm diam x 300 µm high; wall of the conidiomata 12.5-20 µm thick, composed of brown, thick-walled *textura angularis* with cells about 5-8 µm in diam. Conidiophores absent. Conidiogenous cells enteroblastic, phialidic, hyaline, cylindrical, simple, smooth, thin-walled, 7.5-12.5 x 2.5-4.0 µm. Conidia one-celled, hyaline, thick-walled, obovate to ovato-rounded, apex obtuse, base tapered to a distinct protuberant scar, up to 1 µm long, centric or occasionally exocentric towards one side, with small guttules, 15-25 x 10-13.8 (mean = 19.5 x 11.0) µm; mean conidium length/width ratio = 1.77:1. Microconidia one-celled, hyaline, ellipsoid, smooth, thin-walled, two-guttulate, 7-8.5 x 3-4.5 µm.

On dead branches of *Eucalyptus* spp.

Specimens examined: Tasmania, Pine Tree Rivulet, Great Lake, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET44c (with microconidia); Seed orchard, North Forest Products, on *E. delegatensis* R. Baker, 16 April 1996, *Z.Q. Yuan* W19a.

About 17 species in the genus *Cryptosporiopsis* Bubak & Kabat have been described so far (Sutton 1980; Lal and Kapoor 1984; Johnston and Fullerton 1988; Gene *et al.* 1990; Kowalski and Bartnik 1995; Sankaran, Sutton and Balasundaran 1995). None of the known species of the genus adequately accommodate this present species occurring on eucalypt stems. *Cryptosporiopsis eucalypti* Sankaran & B. C. Sutton, a recently described species causing leaf spots and dieback of young eucalypt shoots, is a common species on *Eucalyptus* spp. and has been found in many countries, including Australia, Brazil, India, Japan, Thailand, Vietnam and USA (Ferriera 1989; Old and Yuan 1994; Sankaran, Sutton and Balasundaran 1995). *Cryptosporiopsis* sp. is somewhat similar to *C. eucalypti* but the morphology of its conidia and microconidia is different. Conidia of *C. eucalypti* are more elongated ellipsoid and narrower (4.5-8 µm *fide* Sankaran, Sutton and Balasundaran 1995) than those of *Cryptosporiopsis* sp.. Microconidia of *C. eucalypti* are small (2-3.5 x 1-1.5 µm) and ovoid in shape, not ellipsoid as in *Cryptosporiopsis* sp.. However there is not sufficient evidence as yet to support the description of this *Cryptosporiopsis* sp as a new species.

Fig. 1.4–12: ***Cryptosporiopsis*** **sp.**



Bar = 12.5 µm for **A**; 15 µm for **B** and **C**; 100 µm for **D**

A. Conidia (ET44c);
B. Conidia (W19);
C. Part of conidioma (ET44c);
D. Vertical section of conidioma (ET44c)

5. *Cytospora eucalypticola* van der Westhuizen

South African Forestry Journal 54: 10 (1965)

Conidiomata eustromatic, immersed in outer bark or deeply in stems, separate, multilocular; locules 150-350 μm in diam. Conidiophores hyaline, branched irregularly, up to 20 μm long and 1.5-2 μm wide. Conidia 3.3–5.5 x 0.8–1.2 μm, hyaline, aseptate, thin-walled, allantoid.

On twigs and branches of eucalypts.

Specimens examined: Tasmania: Black Jack's Hill, Little Seanport, on *E. amygdalina,* 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET7b; Bronte Park, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET47; Christmas Hills, on *E. obliqua*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC43; Conara, on *E. amygdalina*, 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC70-72; Fingerpost, Waratah, on *E. nitens*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC25 & WC28; Grass Tree Hill, on *Eucalyptus* sp., 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC80; Henty Bridge, Strahan, on *E. regnans* (?), 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC13,WC14 & WC17; Hobart, on *Eucalyptus* sp. 8 Nov. 1995, *Z.Q. Yuan* 85; Jackeys Marsh, on *E. delegatensis,* 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET37a; Leighlands Rd, Ewandale, on *Eucalyptus* sp., 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC69; Liffey Falls, on *E. nitens*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET39 & ET40; Mawbanna Rd, Mawbanna, on *E. obliqa*, 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC53; Pine Tree Rivulet, on *E. pauciflora*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET42 & ET43; Porter Bridge Rd, Deloraine, on *E. pauciflora*, 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC53; St. Patricks River, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET33; Tayatea Rd, Togari, on *E. nitida*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC49; Tunbridge, on *E. obliqua*, 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC73 & WC74; same location, on *Eucalyptus* sp., 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC76; Tunnack, on on *Eucalyptus* sp., 13 Dec. 1995, *Z.Q. Yuan & M. Hall* WC78; Upper Dromedary, on *E. regnans*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET58; Welcome River, Montagu, on *E. ovata*, 11 Dec. 1995, *Z.Q. Yuan & M. Hall* WC38.

This fungus is common on a wide range of eucalypt species, especially when trees have been stressed. Morphological characteristics fit well with the original description of the species given by van der Westhuizen (1965a). A *Valsa* teleomorph was often associated with this fungus.

6. *Dichomera eucalypti* (Wint.) B.C. Sutton

Mycol. Pap. 138: 182 (1975)

Syn.: *Camarosporium eucalypti* Wint. rev. Mycol. 8: 212 (1886).

(Other synonyms see Sutton, 1975).

Conidiomata pycnidoid to eustromatic, immersed, separate, monolocular, subglobose, 100-180 μm diam, 80-160 μm high. Conidia 10-14.0 x 8.0-10 μm, brown, muriform, euseptate, with 1-3 transverse (often oblique) septa and 1-2 longitudinal or oblique septa, variable in shape, globose, subglobose, obovoid or obpyriform, smooth-walled.

On dead stems of eucalypt.

Specimens examined: Tasmania: St. Patricks River, on *Eucalyptus* sp., 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET32.

The present collection fits well with *D. eucalypti* morphologically in all aspects except for the pycnidioid or monolocular eustromatic conidiomata. As some species in the genus have been reported having both types of conidiomata (Sutton 1980), and also because of the high morphological similarity of the conidia, the present collection is best identified as *D. eucalypti*. This species has been recorded on leaves of *Eucalyptus rubida* in New South Wales and *E. viminalis* in Victoria, Australia (Sutton 1975).

7. *Dinemasporium strigosum* (Pers : Fr.) Sacc.

Michelia 2: 281 (1881)

Syn.: *Peziza strigosa* Pers., Syn. Meth. Fung.: 648 (1801).

(Other synonyms see Sutton, 1980).

Conidiomata cupulate, superficial, aggregated, black, 150-200 μm diam, Setae dark brown to black, 2-6-septate, 150-200 x 5-6 μm. Conidiophores hyaline, cylindrical, 20-25 x 1.5-2.5 μm. Conidia hyaline, aseptate, fusoid, straight or slightly curved, 10-12.5 x 1.5-2.5 μm, with a single unbranched appendage at each end. Appendages 5-10 μm long.

On dead branches of eucalypt.

Specimen examined: Tasmania: Goulds, Hounville, on *E. nitens*, 11 Aug.. 1995, *Z.Q. Yuan* 37.

This fungus has been found commonly on leaves of many graminaceous plants with a world-wide distribution (Sutton 1980; Nag Raj 1993) and as well as on *Acacia catescens* in Australia (Yuan 1996). This is the first record on eucalypt.

8. *Endothiella gyrosa* Sacc. (Fig. 1.3–2b,j; 1.4–10d)

Ann. Mycol. 4: 273 (1906).

Conidiomata pseudostromatic, immersed and erumpent through bark, pulvinate, separate, multilocular, bright orange, 1.5-2.8 mm in diam and up to 2.0 mm high. Conidia 2.0–6.5 x 1.0–1.5 μm, hyaline, aseptate, cylindrical to allantoid, smooth, thin-walled.

On cankered branches of eucalypts.

Specimens examined. Tasmania: Black Jack's Hill, Little Seanport, on *E. amygdalina,* 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET8; Blue Gum Rd, Ridgley, on *Eucalyptus globulus*, 1 Aug. 1995, *Z.Q. Yuan* 73; Buckland Rd, Buckland, on *Eucalyptus* sp., 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET5; Florentine Valley, Westfield, on *E. regnans*, 20 July 1995, *Z.Q. Yuan* 52; Mitchells Creek, Buckland, on *E. globulus*, 5 Dec. 1995, *Z.Q. Yuan & M. Hall* ET3; Nugent, Huonville, on *E. pulchella*, 14 Nov. 1995, *Z.Q. Yuan* 86; Seed orchard, Ridgley, on *E. delegatensis*, 1 Aug. 1995, *Z.Q. Yuan* 66; Ulverstone, on *E. globulus*, 12 Dec. 1995, *Z.Q. Yuan & M. Hall* WC60; Woolnorth, on *E. nitens*, 17 April 1996, *Z.Q. Yuan, C. Mohammed & D. de Little* W3 & W6.

Endothiella gyrosa is the anamorph of *Endothia gyrosa* according to Barr (1978) and widespread in Australia (Old *et al.* 1986). It is the only stage that has been found in Western Australia (Shivas 1989; Shearer 1994). Since it was initially recorded in Australia (Davison and Tay 1983; Walker *et al.* 1985), *Endothiella gyrosa* has always been referred to as *Endothiella* sp. Examination of its morphological features, such as conidioma type and colour, conidial shape and size showed that the Tasmanian collections fit well to *Endothiella gyrosa* as described by Barr (1978).

9. *Harknessia* cf. *eucalypti* Cooke in Cooke & Harkn. (Fig. 1.4–5d; 1.4–7a,b;1.4–10f)
Grevillea 9: 85 (1881).

Conidiomata caulicolous, separate, discrete, pycnidial, immersed, unilocular, subglobose to globose, 400-800 µm diam, ostiole with a furfuraceous margin surrounding the black shining conidial mass. Walls 4-6 cells thick, composed of hyaline to pale brown *textura angularis*. Conidiophores absent. Conidiogenous cells discrete, hyaline, smooth, cylindrical, 8.5-18.5 x 4.5-6.5 µm, formed from the inner cells of the conidiomatal wall, determinate or proliferating. Macroconidia holoblastic, narrowly ellipsoidal, ovoid or broadly ventricose, aseptate, dark brown, smooth, apex obtuse to bluntly apiculate, base truncate, with one or two central globose guttules, 18-35 x 12-16 µm (mean = 25.4 x 13.9 µm) on stems, 15-22.5 x 10-14 µm in culture; basal appendage hyaline, unbranched, 5-20 µm long on stems, 5-18 µm long in culture. Microconidiogenous cells in the same or separate conidiomata, subcylindrical, hyaline, smooth-walled, with cytoplasmic channel and periclinal thickening, 7-12 µm long, and 2-4 µm wide at base. Microconidia holoblastic, apical, hyaline, aseptate, smooth, fusoid (rarely ellipsoidal), 5-10 x 2-4 µm.

On cankered branches of eucalypts.

Specimens examined. Tasmania: Goulds, on *Eucalyptus nitens*, 11 Aug. 1995, *Z.Q. Yuan* 79 (VPRI 20786); Burnie, on *E. nitens*, 1 Aug. 1995, *Z.Q. Yuan* 70 (VPRI 20787); same locality, on *E. delegatensis*, 1 Aug. 1995, *Z.Q. Yuan* 72 (VPRI 20788); Hastings, on *E. nitens*, Aug. 1995, *T. Wardlaw* 83 (VPRI 20789); Westfield, on *E. regnans,* 13 July 1995, *Z.Q. Yuan* 43 (VPRI 20791) and 46 (VPRI 20792); same locality and host, 20 July 1995, *Z.Q. Yuan* 55 (VPRI 20793); same locality and host, 20 July 1995, *Z.Q. Yuan* 60 (VPRI 20822)

The *Harknessia* species is similar to *H. eucalypti* Cooke in conidium morphology and size, except for the presence of a mucilaginous sheath surrounding the conidia. Sutton (1971) and Swart (1972) observed a thin mucus sheath on some developing conidia of *H. eucalypti*. However, this collection showed no evidence of a mucus sheath in either the fresh or old specimens. *H. eucalypti* has been reported on leaves and twigs of *E. globulus* and *E. grandis* in several countries including Australia (Nag Raj 1993;

Sutton 1971, 1980). A teleomorph (*Wuestneia epispora*) and a synmicroconidial state was found associated with this fungus (see Section 1.4.1). If this fungus is *H. eucalypti*, it will be the first record of the teleomorph for *H. eucalypti*.

10. *Neoplaconema cymbiforme* Z. Q. Yuan & C. Mohammed (Fig. 1.4–9e; 1.4–13)
Mycological Research 101: 1533 (1997)

Caulicolous. Conidiomata pseudostromatic, immersed, unilocular, black, ostiolate, 400-500 µm diam; wall composed of thin-walled *textura angularis* (5-8 µm in diam.); loculi 150-200 µm diam, surrounded by pseudoparenchyma of textura intricata intermixed with host tissue. Conidiophores hyaline, 1-2-septate, branched at the base, smooth-walled, invested in mucus. Conidiogenous cells hyaline, discrete or integrated, lageniform to cylindrical, channel minute but periclinal wall considerably thickened, smooth-walled, 5-12.5 x 2.5-4.0 µm. Conidia 15.0-21.3 x 2.5-4.0 (mean 16.9 x 3.1) µm, unicellular, colourless, thin and smooth-walled, naviculate, tapering towards both ends, with an obtuse apex and a narrow truncate base, straight or occasionally slightly curved, bearing an apical appendage of type A; appendage arising as a tubular extension of the conidium body and delimited from it by a septum, unbranched, slightly attenuated, flexuous, 7.5-11.3 (mean 9.6) µm long; mean conidium length/width ratio = 5.45 : 1.

On cankered branch of *Eucalyptus nitens*.

Specimen examined: Tasmania: Basil's Rd, Ridgley, North Forest Products, on *E. nitens* (Deane & Maiden) Maiden, 12 Sept. 1996, *D. de Little* (VPRI 21090), Holotype.

Only a single species, *N. napelli* (Maire & Saccardo) Sutton, has been accepted in the genus *Neoplaconema* Sutton (Sutton 1977; Nag Raj 1993). That fungus was found on stems and branches of *Aconitum napellum* and *A. toxicum* in Germany and Romania (Nag Raj 1993). The conidia of *N. napelli* are shorter but wider than those of *N. cymbiforme*, with a mean conidium length/width ratio of 3.54:1 and long apical appendages up to 27 µm long (*fide* Nag Raj 1993).

The shape and size, as well as the apical appendage length of the conidia in *N. cymbiforme* are similar to those of *Mastigosporella hyalina* (Ellis & Everhart) Höhnel on leaves of *Quercus coccinea* (Nag Raj 1993). However, species of the genus *Mastigosporella* Höhnel have typical pycnidial conidiomata (without surrounding pseudostromatic tissue) and conidiogenous cells proliferating percurrently. Conidia also have apical appendages of type A as in *Neoplaconema*, but not separated from the conidium by a septum.

The teleomorph of *Neoplaconema* is unknown to date. Some immersed, spherical perithecia were found adjacent to or below the conidiomata of *N. cymbiforme* in the cankered area of stems, but no ascospores were observed.

11. *Pestalotiopsis neglecta* (Thuem.) Stey. (Fig. 1.4–10a)

Trans. Brit. Mycol. 36:83 (1953)

Syn.: *Pestalotia neglecta* Thuem., Inst. Rev. Sci. Litt. Coimbra II, 27: 326 (1880).

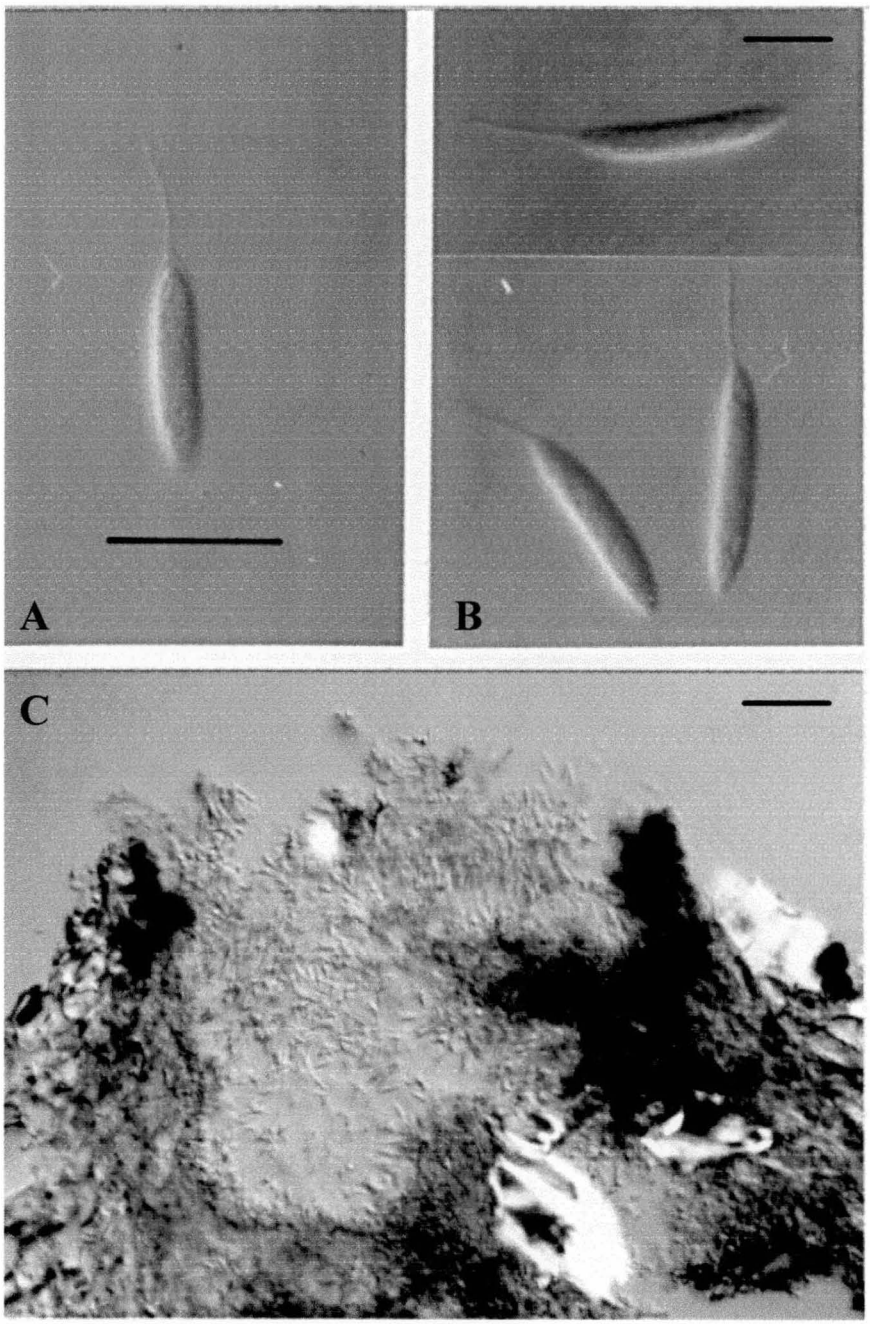
Colony white to off white, dense with centric rings, reverse also white, easily sporulating with black conidial mass (drops) on colony surface either in the dark or when exposed to the light. Conidia narrow fusiform, straight or slightly curved, 4-septate, 20-28 x 5-6.5 µm; intermediate colored cells 14-17.5 µm long, smooth-walled, pale olivaceous, with two upper colored cells often slightly darker than the lowest; apical hyaline cells long cylindric or conic bearing 3 unbranched tubular, 10-25 µm long appendages; basal hyaline cells 5-7 µm long, attenuated into erect basal appendages, 4-10 µm long.

In healthy bark tissue of eucalypt.

Specimens examined. Tasmania: isolated from healthy bark tissue of smooth-barked *Eucalyptus nitens*, 6 July 1995 *Z.Q. Yuan* ECF30,34,35; isolated from healthy bark tissue of rough-barked *E. nitens*, 6 July 1995 *Z.Q. Yuan* ECF31,32, 33.

The present fungus isolated from eucalypt bark fits well with *P. neglecta* in respect to conidial and appendage morphology (Guba 1961). As it was isolated from plant tissue, there is no comparative information on the morphology of natural conidiomata.

Fig. 1.4–13: ***Neoplaconema cymbiforme***



Bar = 15 µm for **A**; 8 µm for **B**; 45 µm for **C**

A, B. Conidia;
C. A vertical section of conidioma

12. *Phoma* sp.

Colonies white initially, fluffy, turning to dark or blackish gray, with flocculose aerial mycelium and concentric rings, reverse orange coloured at first, then becoming dark brown to olive brown, with radial lines, no change in colony colour when exposed to light. In culture, conidiomata produced on the surface of agar are brown to black, irregular-shaped; walls are composed of thin-walled, *textura angularis*. Condiophores present, 20-37.5 x 1.5-2 μm, filiform, septate. Conidia 5.5-8.75 x 2-3.0 μm, hyaline, one-celled, ellipsoid to short cylindrical, some conidia slightly attenuated at both ends, thin-walled, guttulate.

On cankered stems of eucalypts.

Specimens examined. Tasmania: Bothwell, on *E. rubida* Dean & Maiden, 1 Dec. 1995, *T. Wardlaw* 47 (ECF54).

More than 2000 species have been described in the genus *Phoma* Sacc. There is no up to date taxonomic monograph available for identifying species in the genus. Sutton (1980) included 29 species in a key. Among them, conidiophores were observed only in *P. cava* Shulz. and *P. tracheiphila* (Petri) Kantachveli & Gikachvili and only the former has filiform conidiophores as in the present fungus. However, conidia of *P. cava* are small (3-4 x 1-2 μm) and colony morphological characteristics, such as colour and density are also different (Sutton 1980).

Pathogenicity of *Phoma* sp. has been investigated by artificial inoculation tests on both one-year-old seedlings and 16-year-old plantation trees of *Eucalyptus nitens* (Chapter 2). The *Phoma* species was able to produce large sunken canker lesions on inoculated seedlings and cause seedling mortality. In view of its demonstrated pathogenicity, further investigation of its taxonomic status at species level is needed.

13. *Seiridium eucalypti* Nag Raj (Fig. 1.4–10b)

Coelomycetous Anamorphs with Appendage-bearing Conidia: 862 (1993)

Conidiomata acervular, immersed to erumpent, separate or occasionally gregarious, 200-850 μm diam. Conidiophores hyaline, cylindrical, 20-40 x 3-3.8 μm. Conidia cylindric-fusoid, mostly slightly curved, with 5 transverse septa, 20.5-35.5 x 6.0-11.0 μm, 4 intermediate coloured cells 20-25.5 μm long, brown to dark brown, concolourous, walls thick and darker, slightly constricted at the septa; apical and basal cells hyaline, conical, apical appendages unbranched, 7.5-22.5 μm long, basal appendages unbranched, in the centre of the base of conidia or towards the side of conidia with a truncate base, 2.5-10 μm long.

On cankered branches of eucalypts.

Specimens examined. Tasmania: Basil's Rd, Ridgley, on hybrid of *E. camaldulensis* and *E. nitens*, 12 Nov. 1996, *Z.Q. Yuan* 300; same location on *E. nitens*, 6 May 1997, *K.M. Old* 310; Navarre Plains, Dervent, on *E. obliqua*, 11 Dec. 1995, *Z. Q. Yuan & M. Hall* WC3; Trafalgar Flant, St. Helens, on *E. viminalis*, 6 Dec. 1995, *Z.Q. Yuan & M. Hall* ET17b; Westfield, on *E. regnans,* 13 July 1995, *Z.Q. Yuan* 50; same locality and host, 20 July 1995, *Z.Q. Yuan* 54.

Although it was originally proposed on leaves of a *Eucalyptus* species from South Australia (Nag Raj 1993), *S. eucalypti* appeared fairly common on branches or stems of *Eucalyptus* spp. in Tasmania.

Apart from the first collection of *S. eucalypti* on branches of *Eucalyptus delegatensis* R. Baker in Tasmania in 1987 (Yuan and Old 1995), a number of additional collections were made on a range of eucalypt species in northern Tasmania during the present survey. Some of them were collected from the same locality as the first collection. Morphological examinations showed they are all identical to each other.

14. *Seiridium papillatum* Z.Q. Yuan (Figs. 1.4–9i; 1.4–14; 1.4–15)
in Yuan and Mohammed, Australian Systematic Botany 10: 70 (1997)

Caulicolous. Conidiomata stromatic, acervuloid, scattered, immersed to erumpent, round to elongated, 200-1000 µm wide, 60-120 µm deep, dehiscing by one to three splits in the overlaying host tissue and covered with pulverulent black masses of conidia. Basal stroma up to 75 µm, of *textura angularis*, cells thick-walled, pale brown, paler toward the conidial hymenium. Conidiophores septate, smooth, branched at base, 65-125 µm long, invested in mucus. Conidogenous cells discrete, cylindrical, hyaline, smooth, 11-17.5 x 2-2.5 µm, with 1 proliferation. Conidia ellipsoid to sub-cylindrical, straight, sometimes slightly curved, 5-septate (rarely 4-7- septate), with slight constrictions at the septa, septal pores invisible, 26.5-34.5 x 10-15 (mean = 31.5 x 12.5) µm, bearing appendages; basal cell obconic, or ob-trapezoid with a truncate base, hyaline, thin-walled, smooth, 2-3 µm (mean = 2.1 µm) long, 4 median cells short cylindrical, brown to dark brown, smooth-walled or faintly striate in Shear's mounting medium, thick-walled, together 21-29.5 µm (mean = 27.0 µm) long (second cell from base 6-8.5 (mean = 7.9) µm; third cell 5.2-7.1 (mean = 6.3) µm; fourth cell 5.2-7.1 µm (mean = 6.3 µm); fifth cell 5.5-7.5 (mean = 6.7) µm); apical cell conical, hyaline, thin-walled, smooth, 2-3.5 (mean = 2.4) µm long; appendages papillate, unbranched; apical appendages 1.25-2.5 (mean = 1.7) µm long; basal appendages often present, centric, up to 2.0 µm long; mean conidium length/width ratio = 2.52:1. Microconidial synanamorph not observed.

On stems of *Eucalyptus* spp.

Specimens examined. Tasmania: Seed orchard, North Forest Products, Burnie, on *Eucalyptus delegatensis*, 1. VIII. 1995, *Z. Q. Yuan* 64 (Holotype VPRI 20825); Tarraleah, on *Eucalyptus* sp., 6. XII. 1995, *Z. Q. Yuan & M. Hall* ET54 (VPRI 20826); ECF56, culture derived from holotype stored as dried agar sheet (VPRI 20827).

Maximum rates of growth of *S. papillatum* were recorded at 25 °C. Average radial growth rate of colonies on MEA in the dark at this temperature was 1.2 mm per day (Fig. 1.4–16).

Colony colour varied slightly from snow-white to off-white, velvety, with light brown to olive brown or dark brown reverse, when subjected to light changing pinkish-white to pink. Colonies show a sectoring growth pattern with small lobes at margins. After incubation under light (4 weeks on MEA; 2 weeks on wheat-rice bran) acervuli and conidia were produced at the margin of the agar and on bottom or wall of flasks.

Conidia produced in culture vary in size and cell number. Most are typically 5-septate and ellipsoid to sub-cylindrical in shape, and occasionally 4-, 6- or 7-septate; 5-septate conidia 25.0-37.5 x 10-14 (mean = 33.5 x 12.0) µm, 4 intermediate coloured cells 20-27.5 (mean = 23.5) µm long, 6-7-septate conidia up to 45.0-47.5 x 10-12.5 µm in size; apical appendages up to 2.0 µm long, basal appendages mostly 1 µm long; mean conidium length/width ratio = 2.79:1 (for 5-septate conidia) and 4.1:1 (for 6-7-septate conidia).

Seiridium papillatum can be distinguished by its short papillate conidial appendages and its large, ellipsoid to sub-cylindrical, striate conidia. Based on Nag Raj (1993), several species have conidia with appendages less than 5 µm long, but only *S. canariense* (Petrak) Nag Raj & Kendrick, *S. cardinale* and *S. intermedium* (Saccardo) Sutton are morphologically similar to *S. papillatum*.

To illustrate the main points of separation, a key to *S. papillatum* and closely related species of the genus is provided. Because of marked morphological variations under cultural conditions, features of *S. papillatum* used in the key are based on field specimens. For other known species features are based on descriptions given by Nag Raj (1993).

Key to Seiridium papillatum and similar Seiridium species

1. Median cell walls striate 2
1. Median cell walls smooth 4

2. Appendages more than 5 µm long
2. Appendages less than 5 µm long 3

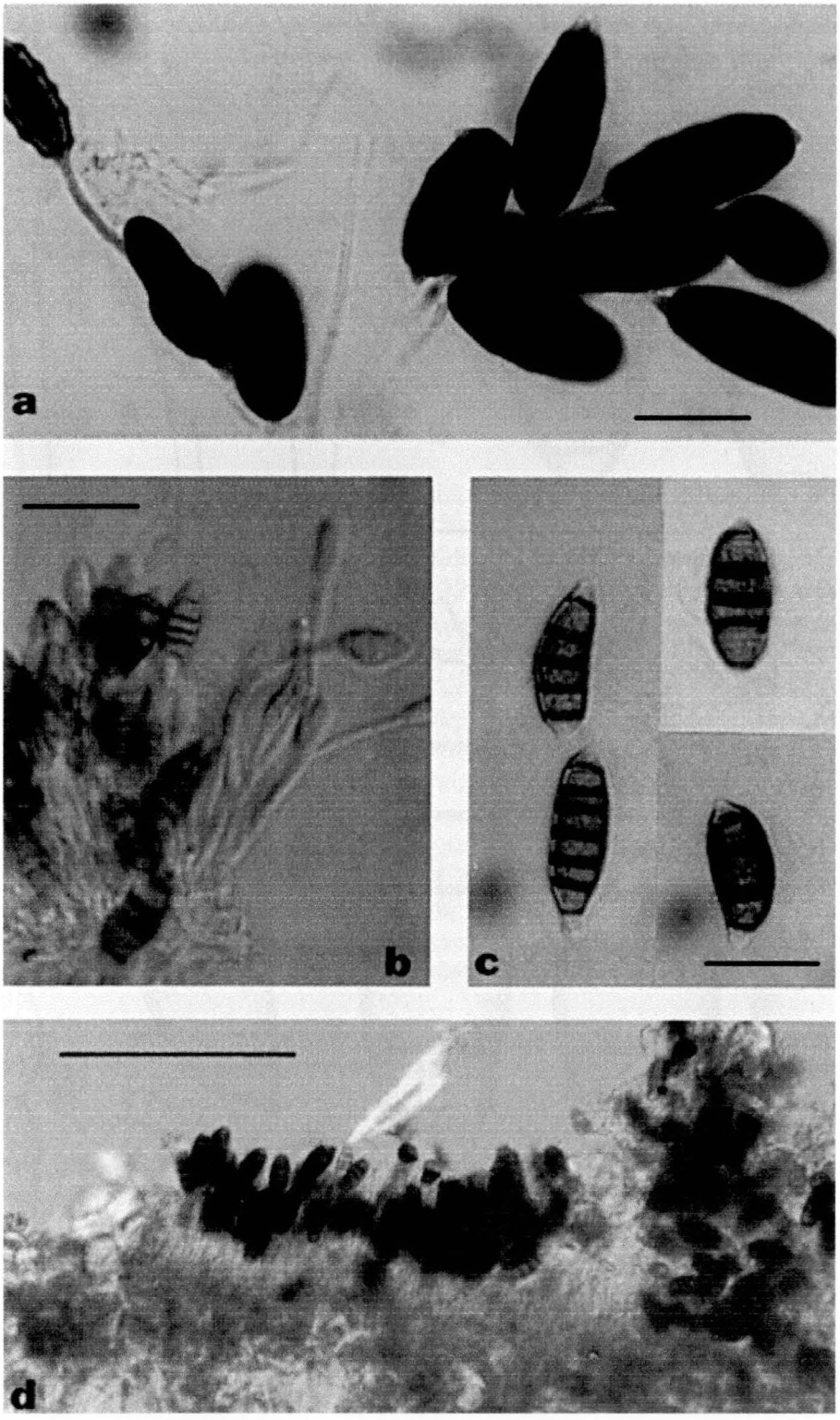
3. Appendages corniform, mean apical appendage length 2.2 µm; conidiogenous cells with 2-3 proliferations; conidia fusiform, not constricted at septa, mean size of conidia 28.5 x 10 µm; total mean length of median cells 21.2 µm; mean conidium length/width ratio = 2.85:1 ..... *S. intermedium*

3. Appendages papillate; mean apical appendage length 1.7 µm; conidiogenous cells with 1 proliferation; conidia ellipsoid, constricted at septa, mean size of conidia 31.5 x 12.5 µm; total mean length of median cells 27.0 µm; mean conidium length/width ratio = 2.52: 1 ..... *S. papillatum*

4. Mean conidium length/width ratio > 3:1; mean conidium width 9 µm or less; mean apical appendages more than 10 µm long ..... *S. eucalypti*
4. Mean conidium length/width ratio < 3:1; mean conidium width more than 9 µm; mean apical appendages less than 5 µm long 5

5. Conidiomata acervuloid, up to 1000 µm wide, 120 µm deep; conidiophores arise at base and sides of conidiomata; conidiogenous cells with 1 proliferation; septal pores not visible ..... *S. papillatum*
5. Conidiomata pycnidioid, up to 400 µm wide, 230-300 µm deep; conidiophores arise all around cavity of conidiomata; conidiogenous cells with 4-6 proliferations; septal pores distinctly visible 6

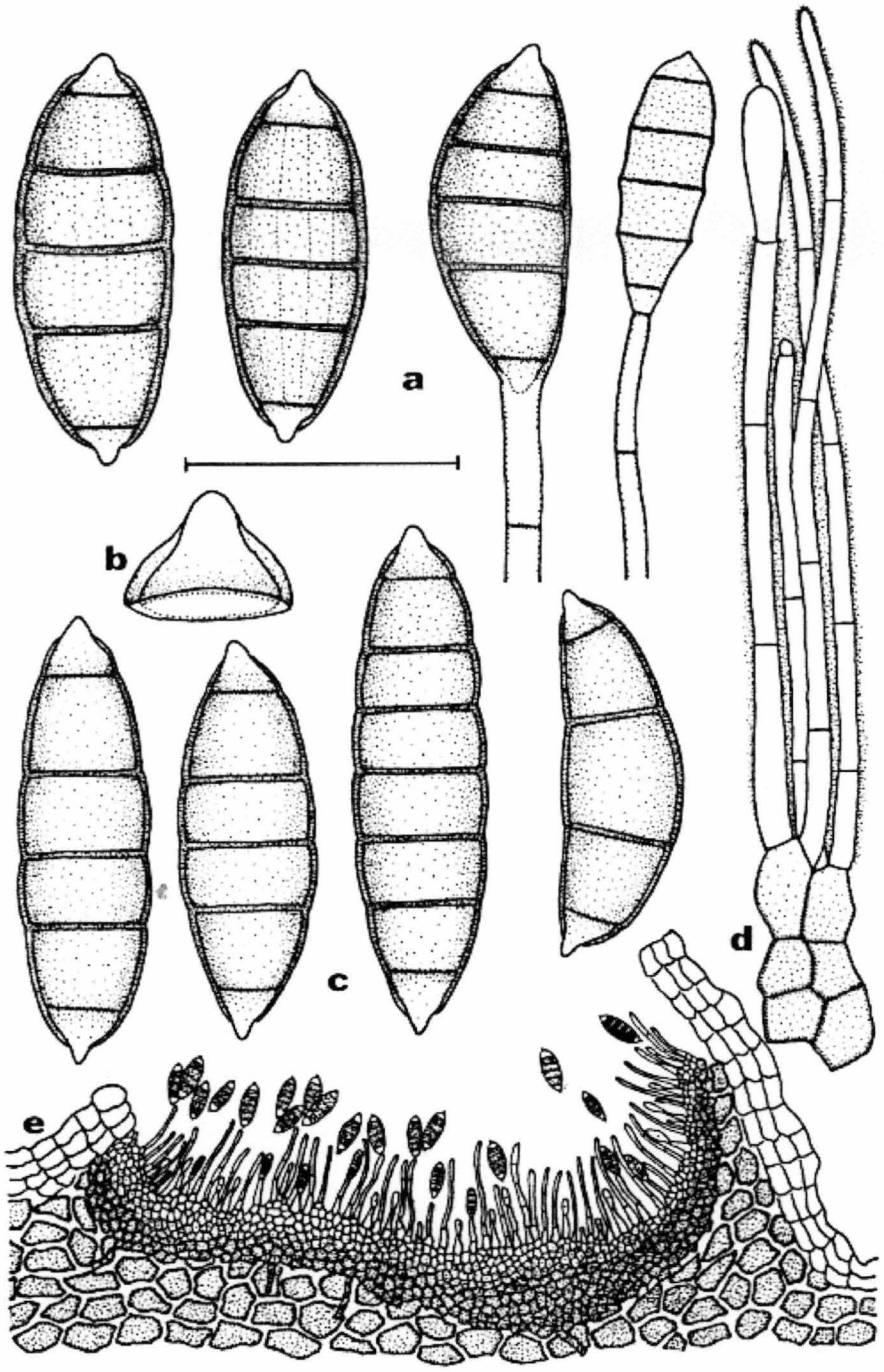
6. Second and fifth cells of conidium longer than third and fourth cells; mean conidium size 28.5 x 12 µm with a length/width ratio of 2.4:1; apical appendages 1.5-5 µm long ..... *S. canariense*
6. Second cell of conidium longer than third, fourth and fifth cells; mean conidium size 25.5 x 9.3 µm with a length/width ratio of 2.7:1; apical appendages 0.5-1.5 µm long ..... *S. cardinale*



Bar = 15 µm for **A**; 30 µm for **B**; 25 µm for **C**; 200 µm for **D**

Fig. 1.4-14: ***Seiridium papillatum***

A. Conidia on stem of eucalypt; **B.** Developing conidia and conidiophores on cultures; **C.** Conidia on cultures (note the 4- and 7- septate conidia); **D.** Conidioma in vertical section



Standard line = 25 µm for **a, c** and **d**; 12.5 µm for **b**; 250 µm for **e**

Fig. 1.4–15: ***Seiridium papillatum***

A. Conidia; **B.** Apical cell with papillate appendage; **C.** Conidia from culture; **D.** Conidiophores; **E.** Conidioma in vertical section.

As listed in the key, both *S. canariense* and *S. cardinale* differ from *S. papillatum* in (*i*) type and size of conidiomata; (*ii*) origin of conidiophores and the number of proliferations of conidiogenous cells; (*iii*) presence of septal pores and (*iv*) lack of striations on conidia.

Seiridium cardinale also can be distinguished by its typical fusiform conidia (21-30 x 8-10 µm) in contrast to the ellipsoid conidia (26.5-34.5 x 10-15 µm) of *S. papillatum*. So far *S. cardinale* has been found only on conifers, not hardwoods.

Conidium size in *S. papillatum* and *S. canariense* appear to overlap slightly. However, in addition to differences (*i*) to (*iv*) mentioned above, the conidial appendages of *S. canariense* are longer and much more slender than those of *S. papillatum*.

Within the group of known species with striated conidia in the genus, *S. papillatum* is morphologically close to *S. intermedium*. However, *S. intermedium* conidia are typically fusiform in shape and much shorter and narrower than those of *S. papillatum*.

Conidia of *S. papillatum* formed in culture were variable in both size (25.0-47.5 x 10-14 µm) and septation (4-7 septa). This is the first report of 4- and 7-septate conidia in the genus. Usually species of *Seiridium* have 5-septate conidia. Two *Seiridium* species, *S. turgidum* (Atkinson) Nag Raj and *S. unicorne* (Cooke & Ellis) Sutton were reported occasionally as having 6-septate conidia in field specimens (Nag Raj 1993).

Characteristics in artificial culture can be extremely important taxonomic features for many fungal genera, such as *Alternaria* Nees, *Colletotrichum* Corda, *Fusarium* Link and *Phoma* Sacc.. Separation of species within these genera is mainly based on morphology under certain aseptic conditions.

Variation in conidial morphology observed in *S. papillatum* cultures may suggest that conidium size and cell number for other species in this genus could also be influenced by cultural conditions. Such cultural features could affect species classification within the genus. In order to compare cultural characteristics of *S. papillatum* with those of

other species in the genus, it would be necessary for all isolates to be available and cultured under uniform conditions.

The cultural characteristics of *S. papillatum*, such as colony morphology and growth rate at different temperatures, were compared with two other *Seiridium* species; *S. eucalypti* and *S. unicorne* isolated from *Callitris* sp. Colony characteristics of *S. papillatum* on MEA are similar to that of *S. eucalypti* and *S. unicorne* from *Callitris*, although the colony colour of *S. unicorne* is slightly different (off white to mouse-gray) in darkness. When exposed to light, all three cultures became pinkish white to pink. Pink colouration of colonies growing under light may be a general feature for the genus, as at least two species have been reported previously producing salmon to apricot colony when grown under illumination (Boesewinkel 1983; Yuan and Old 1995).

The colony diameter of cultures grown on MEA in darkness at different temperatures for *S. papillatum*, *S. eucalypti* and *S. unicorne* is shown in Fig. 1.4–16. The temperature for the maximum growth of *S. eucalypti* and *S. unicorne* was 20 °C, whereas that for *S. papillatum* was 25 °C. At 25 °C the mean colony diameter for *S. papillatum* was significantly higher than that of *S. eucalypti* and *S. unicorne* ($P = 0.05$). This suggests that *S. papillatum* may be more tolerant of higher temperatures than both *S. eucalypti* and *S. unicorne* although the growth of all three fungi was strongly inhibited at 30 °C.

Preliminary DNA investigation of Seiridium species

Seiridium is a well-documented fungal genus of mitosporic coelomycetes. Earlier accounts of this genus are found in Guba (1961) and Sutton (1969, 1975, 1980). Nag Raj (1993) comprehensively described 19 species based on examinations of either holotypes or isotypes and listed 9 uncertain species among which 4 were treated as synonyms of the accepted taxa. To date four *Seiridium* species have been recorded in Australia. They are *S. cardinale*, *S. unicorne* which were found on *Cupressus* spp. (Purnell 1960; Sutton and Gibson 1972), and *S. eucalypti* and *S. papillatum* which were found on *Eucalyptus* spp. (Nag Raj 1993; Yuan and Old 1995).

Fig. 1.4-16: Mean colony diameter of *Seiridium eucalypti*, *S. papillatum* and *S. unicorne* on malt extract agar after 25 days incubation at various temperatures

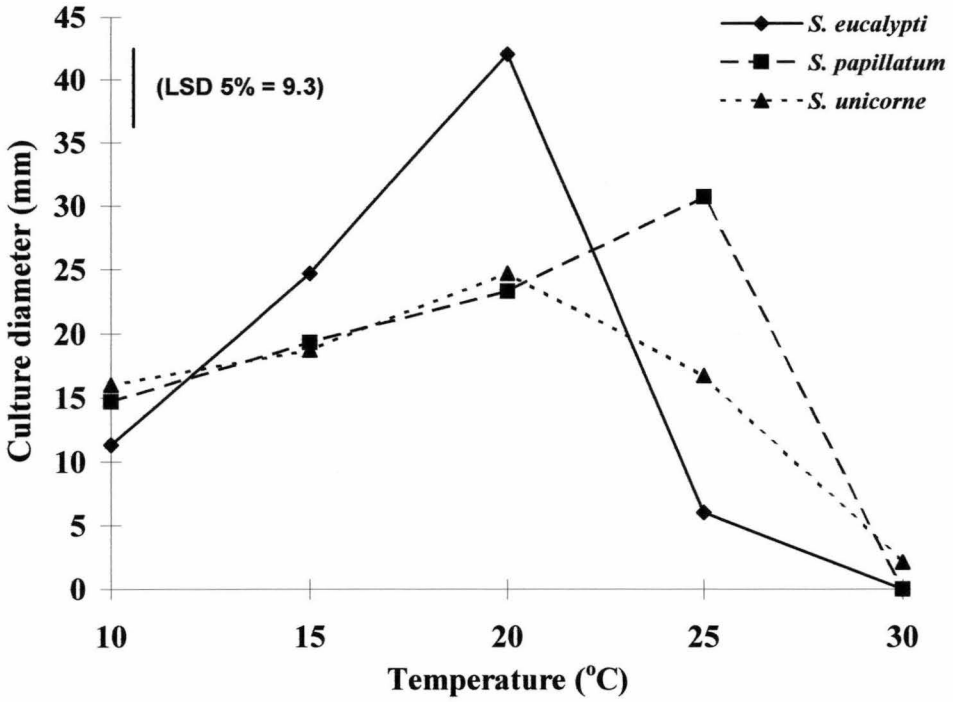
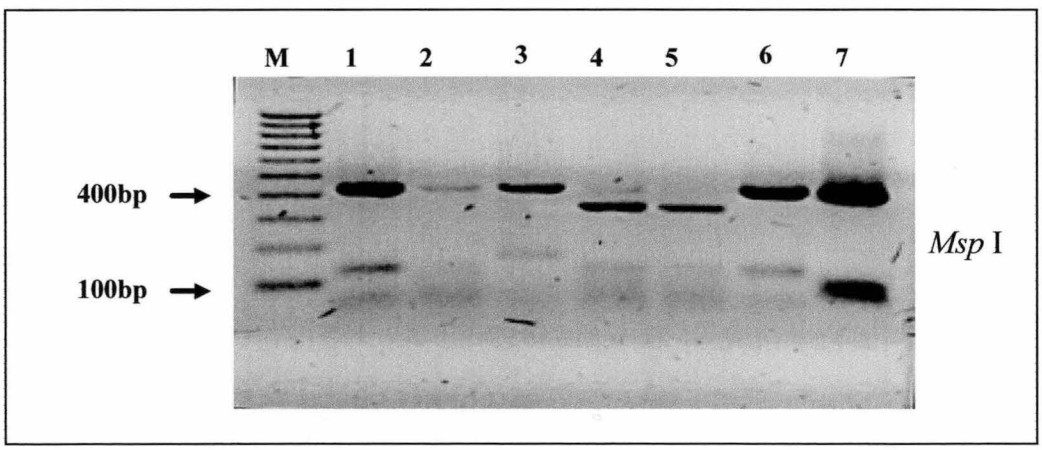


Fig. 1.4–17: Gel electrophoresis of *Msp* I digested amplified ITS of *Seiridium* spp.



Lane 1; *S. cardinale* (ATCC 38654), 2; *S. cardinale* (Castellino), 3; *S. unicorne* (S1), 4; *S. eucalypti* (ECF149), 5; *S. eucalypti* (ECF115), 6; *Seiridium* sp. (Perth), 7; *S. papillatum* (ECF 139)

Markers (M) are 100-bp DNA ladder.

Table 1.4-3: Estimated restriction fragment sizes (rounded to the nearest 10 bp) following digestion with *Msp* I of PCR-amplified internal transcribed spacer (ITS) region of *Seiridium* spp.

	S. cardinale (ATCC386540)	*S cardinale* (Castellino)	*S unicorne* (S1)	*S eucalypti* (ECF115)	*S. eucalypti* (ECF115)	*Seirdium* sp. (Perth)	*S. papillatum* (ECF139)
Uncut	580	580	620	600	600	570	540
Msp I	440	440	440	350	350	420	410
	140	140	180	150	150	150	130
				100	100		

Since the separation of these species is mainly based on the morphology of conidial appendages, there is some disagreement regarding the taxonomy of *Seiridium* species on cypress. Some authorities accepted three species, *S. cardinale*, *S. cupressi* (Guba) Boesewinkel and *S. unicorne* associated with cypress cankers (Boesewinkel 1983; Graniti 1989). Others accepted only two or even one species (Swart 1973; Sutton 1980; Chou 1989; Nag Raj 1993; Viljoen *et al.* 1993). Swart (1973) suggested *S. cardinale* and *S. unicorne* [as *Monochaetia unicornis* (Cooke & Ellis) Sacc.] were one species. Chou (1989) was unable to separate *S. cupressi* from *S. unicorne* based on morphological and cultural variation. Nag Raj (1993) recognised *S. cardinale* and *S. unicorne* on cypress and reduced *S. cupressi* to synonymy with *S. unicorne*. Viljoen *et al.* (1993) compared isolates of these three *Seiridium* species on cypress using DNA sequence analysis and concluded that *S. cupressi* and *S. unicorne* are synonyms of *S. cardinale*. However, this has not been accepted by most European authorities (Spanos *et al.* 1997; Moricca 1997, pers. comm.).

Morphologically, *S. eucalypti* is similar to some long-appendaged species of *Seiridium* found on cypress. Yuan (1989) referred it as *S. cupressi*. More recently Parbery of Melbourne University (pers. comm., 1995) pointed out that *S. eucalypti* is remarkably like *S. unicorne* (as *M. unicornis*), the anamorph of *Lepteutypa cupressi* (Natrass, Booth & Sutton) Swart found in Australia (Swart 1973). He suspected *S. eucalypti* could be the same fungus as the one on cypress and could infect both angiosperms and gymnosperm hosts.

A preliminary molecular analysis of DNA polymorphism was conducted to confirm the separate identities of the eucalypt *Seiridium* species *S. eucalypti* and *S. papillatum,* and to compare these species with other *Seiridium* species on cypress. Polymorphism was assessed by RFLP analysis of the PCR amplified ITS region of rDNA.

Isolates of five *Seiridium* species were tested (Table 1.4-4).

Table 1.4–4: *Seiridium* isolates

Species	Isolate No.[A]		Collection Site	Host
S. cardinale	ATCC 38654		?, Italy	*Cupressus macrocarpa*
S. cardinale	–		Castellino, Italy	*Cupressus macrocarpa*
S. unicorne	S1		Canberra	*Callitris* sp.
S. eucalypti	CBS 343.97	(ECF149)	Westfield, Tasmania	*Eucalyptus regnans*
S. eucalypti	ECF115	(CF39)	Ridgley, Tasmania	*Eucalyptus delegatensis*
Seiridium sp.	–		Western Australia	*Cupressus macrocarpa*
S. papillatum	CBS 340.97	(ECF139)	Ridgley, Tasmania	*Eucalyptus delegatensis*

[A]All ECF isolates are held by the authors at CSIRO, Forestry & Forest Products, Tasmania, and the isolates with CF designation at CSIRO, Forestry & Forest Products, Canberra. ATCC = American Type Culture Collection at Maryland, USA; CBS = Centraalbureau voor Schimmelcultures at Baarn, Netherlands.

The ITS1 and ITS4 primers used to amplify the internal transcribed spacers revealed size polymorphism phenotypes, albeit minor.

This variation in amplified products supported by differences in restriction endonuclease analysis with *Msp* I contradict Parberry's suggestion of similarity between *S. unicorne* and *S. eucalypti* (Fig. 1.4-17 and Table 1.4-3).

The two eucalypt *Seiridium* species, *S. eucalypti* (lane 4 & 5) and *S. papillatum* (lane 7) were also differentiated by this preliminary analysis.

The *Seiridium* sp. collected on *Cupressus macrocarpa* from Perth, Western Australia is morphologically different from *S. cardinale* and *S. unicorne* with long conidial appendages (Yuan unpublished data). Minor differences in amplified product and restriction analysis were detected in comparison to *S. cardinale* and *S. unicorne.*

Three restriction enzymes, *Alu* I, *Hae* III and *Hinf* I were also included in the present investigation and allowed discrimination between *Seiridium* species on conifers and eucalypts. However digestions with these three restriction enzymes were incomplete and must repeated before correct estimation of restriction fragment sizes is possible.

Although the results are preliminary and require further investigation (DNA sequence analysis), they clearly indicate that *S. eucalypti* and *S. papillatum* are separate species within the genus.

15. *Thyrostroma eucalypti* Z.Q. Yuan & Old,

Mycological Research 94: 573 (1990)

Conidiomata acervular or pulvinate, separate, 500-1000 µm diam. Conidiophores subhyaline, short cylindrical, 7-30 x 4-5 µm. Conidia 40.5-68.0 x 17-22.5 µm, obovate or clavate, muriform, with 5-9 transverse and 2-7 longitunidal eusepta, slightly constricted at the septa, brown, smooth-walled, obtuse at the apex and truncate at the base. Paraphyses filiform to geniculate, septate, hyaline, 30-70 x 2-3 µm.

On dead branches of eucalypts.

Specimens examined: Tasmania: Buckland Rd, Buckland, on *Eucalyptus* sp., 4. Dec.. 1995, *Z. Q. Yuan & M. Hall* ET4; Grass Tree Hill, on *Eucalyptus* sp., 13. Dec.. 1995, *Z. Q. Yuan & M. Hall* WC79; Saxelby Creek, St. Helens, on *E. amygdalina*, 4. Dec.. 1995, *Z. Q. Yuan & M. Hall* ET14; Seed orchard, North Forest Products, on *E. delegatensis* R. Baker, 1 Aug. 1995, *Z.Q. Yuan* 65, 75; Sterling River, Rosebery, on *Eucalyptus* sp. 12. Dec.. 1995, *Z. Q. Yuan & M. Hall* WC23; Yorkys Creek, Scamander, on *E. regnans*, 4. Dec.. 1995, *Z. Q. Yuan & M. Hall* ET10.

Thyrostroma eucalypti was established by Yuan and Old (1990) and is commonly found on stems of eucalypts in natural forest in south-eastern Australia. During this survey, *T. eucalypti* was also found on several species of eucalypts in Tasmania. The morphological characteristics comply with the original description of the species given by Yuan and Old (1990), except that the conidia of the Tasmanian collections are slightly larger in size.

16. *Zythiostroma* sp. (Fig. 1.4–10e)

Conidiomata eustromatic, immersed at first, superficial finally, separate, subglobose to collabent, 700-1000 µm diam, 300-400 µm high, dark reddish brown, multilocular, locules 80-200 µm diam; walls 20-35 µm thick, composed of brown to reddish brown, thick-walled *textura angularis*. Ostiole single, papillate, 50-100 µm diam, 100-150 µm high. Conidiophores 17.5-32.5 x 1-2 µm, cylindrical, hyaline, septate and slightly branched at the base. Conidia 2.5-3.5 x 1 µm, hyaline, aseptate, slightly curved, thin-walled, eguttulate.

On cankered and dead branches of eucalypts.

Specimens examined: Tasmania: Mathinna Plains, St. Helens, on *E. ovata*, 5 Dec. 1995, *Z. Q. Yuan & M. Hall* ET21; Navarre Plains, Dervent, on *E. obliqua*, 11 Dec. 1995, *Z. Q. Yuan & M. Hall* WC4a; Oldina Rd, Wynyard, on *E. obliqua*, 12 Dec. 1995, *Z. Q. Yuan & M. Hall* WC58; Port Latta, on *Eucalyptus* sp., 11 Dec. 1995, *Z. Q. Yuan & M. Hall* WC33; Seed orchard, North Forest Products, on *E. delegatensis* R. Baker, 16 April 1996, *Z.Q. Yuan* W19b; Tayatea Rd, Togari, on *E. nitida*, 12 Dec. 1995, *Z. Q. Yuan & M. Hall* WC48b; Wages Rd, Surrey Hill, on *E. nitens*, 18 April 1996, *Z. Q. Yuan* W13; West Ridgley, on *E. nitens*, 18 April 1996, *Z. Q. Yuan* W22c.

The distinctive superficial, reddish brown coloured and multilocular conidiomata indicates that this fungus is best accommodated in the genus *Zythiostroma* Höhn.. The measurements of conidia size match that of *Z. mougeotii* (Fr.) Höhn., but the latter has smaller, red-coloured conidiomata with irregularly arranged locules (Sutton 1980). It is similar to *Z. pinastri* (Karst.) Höhn. in colour of conidiomata and in morphology of conidia, except for the conidia in *Z. pinastri* which are slightly longer (4-6 x 1 µm, Sutton 1980). *Z. pinastri* is a common species on conifers, especially on branches of many *Pinus* species and has been recorded in Asia, Europe and North America (Booth 1959). A further detailed comparison of the present fungus on eucalypts with *Z. pinastri* is needed.

1.4.3 KEY TO SPECIES

The following key to the species found during the survey is proposed to allow quick identification of stem fungi on eucalypts. However, this key will require validation by wide-ranging field tests.

1 Produce ascospores developed endogenously in asci ..... **Ascomycota** (2)

1 Produce conidia in/on conidiomata ..... **Mitosporic fungi** (16)

2(1) Ascospores 1-celled 3

2(1) Ascospores 2- or more-celled 9

3(2) Ascospores allantoid 4

3(2) Ascospores other than allantoid in shape 6

4(3) Stroma bright-colored, diatrypoid (necks straight);
Stroma bright orange internally and externally, becaming rusty brown in the surface with age; ascospores hyaline, one-celled, fusiform to cylindrical, slightly curved, 5.5–12.0 x 1.0–2.0 µm ..... *Endothia gyrosa*

4(3) Stroma black, valsoid (necks converge) or eutypoid (necks straight) 5

5(4) Stroma valsoid, immersed in stems with ostiolar necks of perithecia converging through bark; ascospores hyaline, one-celled, 7–9 x 1.5–2.0 µm .... *Valsa ceratosperma*

5(4) Stroma eutypoid, wide-spreading on surface of stems with ostiolar necks of perithecia erumpent separately; ostioles 3–5 (mostly 3) sulcate; ascospores pale brown, 6–8 x 1.8–2.5 µm ..... *Eutypa spinosa*

6(3) Ascospores brown to dark brown; perithecia superficial, gregarious;
ascospores ellipsoid to broad ellipsoid, with a longitudinal germ slit, 10–13 x 7–8 µm ..... *Coniochaeta pulveracea*

6(3) Ascospores hyaline; perithecia immersed, scattered or in groups 7

7(6) Perithecia scattered, without surrounding stromatic tissue; ascospores thin-walled;
perithecia 300–500 x 250–400 µm; ascospores hyaline, elliptic-cylindric to fusoid-cylindric, with 1 or 2 oil drops, granular, 30.0–47.5 x 8.8–12.5 µm ..... *Phomatospora macrospora*

7(6) Perithecia in groups, occasionally scattered, with surrounding stromatic tissue; ascospores thick-walled 8

8(7) Asci cylindrical to subcylindrical, 100–150 x 12.5–17.5 µm, with a distinct campanulate apical apparatus; ascospores 17.5–27.5 x 6.5–10 µm, elliptic-fusoid to obovoid, without any gelatinous sheath ..... *Wuestneia campanulata*

8(7) Asci clavate, 100–188 x 13–33 µm, without apical apparatus; ascospores 20–35 x 9–15 µm, with a thick, tenacious gelatinous sheath ..... *Wuestneia epispora*

9(2) Ascospores 2-celled 10

9(2) Ascospores 3- or more-celled 13

10(9) Ascomata superficial on stems 11

10(9) Ascomata immersed in stems 12

11(10) Ascomata hysterothecioid, elongate, sometimes branched or star-like, opening by a slit; asci ovate, sessile, 8-spored; ascospores hyaline, ellipsoid, 2-celled, 8.0–11.5 x 3.5–5.0 µm..... *Aulographina eucalypti*

11(10) Ascomata perithecioid, separate to densely gregarious, seated on dark brown subiculum, surface marked with coarse warts, without ostioles; asci with an apical ring; ascospores 27.5–37.5 x 4.5–7.0 µm, constricted at the septum and surrounded by a thin gelatinous sheath ..... *Bertia antennaroidea*

12(10) Perithecia immersed in a stroma outlined by a blackened zone; beak separately protruding through bark; ascospores 10–15 x 3–5 µm, fusoid, constricted at the septum, bearing a delicate setose appendage at each end ..... *Diaporthe fusispora*

12(10) Perithecia immersed in a poorly developed stoma without blackened zone; ascospores 20–35 x 5–7.5 µm, oblong-ellipsoid to cylindrical, constricted at the middle, curved, surrounded by a thin gelatinous sheath .... *Cryptodiaporthe curvata*

13(9) Ascospores with transverse septa only 14

13(9) Ascospores with transverse and longitudinal septa 15

14(13) Ascomata perithecia, superficial, gregarious to crowded on blackened substrate; ascospores 17–21 x 4–6 µm, brown, ellipsoid to fusoid, 3-septate, constricted at the first-formed septum ..... *Melanomma pulvis-pyrius*

14(13) Ascomata apothecia, immersed to erumpent, solitary or 2–5 gregarious; ascospores 62–88 x 2–3 µm, hyaline, 2–5-septate ..... *Therrya eucalypti*

15(13) Ascomata immersed in valsoid groups; ascospores 27–52.5 x 10–18 µm, yellowish brown to dark brown, end cells pale, ellipsoid-fusoid, with 5–13 transverse septa and 3–5 longitudinal septa, constricted at first-formed septum ..... *Fenestella media*

15(13) Ascomata immersed in a thin blackened clypeus; ascospores 17.5–22.0 x 7.5–10 µm, brown to dark brown, ellipsoidal to fusoid with obtuse ends, with 3 transverse septa and 1–3 longitudinal septa ..... *Karstenula ceanothi*

16(1) Conidia with appendages or setulae 17

16(1) Conidia without appendages 24

17(16) Conidia pigmented 18

17(16) Conidia hyaline 21

18(17) Conidia aseptate, with only a basal appendage;
Conidia narrowly ellipsoidal, ovoid or broadly ventricose, dark brown, 18–35 x 12–16 µm; basal appendage unbranched, 5–20 µm long; microconidia hyaline, aseptate, fusoid, 5–10 x 2–4 µm ..... *Harknessia* cf. *eucalypti*

18(17) Conidia septate, with both apical and basal appendages 19

19(18) Conidia 4 septate, with several apical appendages;
Conidia narrow fusiform, 20-28 x 5-6.5 µm; intermediate three cells pale olivaceous, with two upper cells often slightly darker than the lowest; 3 apical appendages unbranched, tubular, 10-25 µm long; basal appendages, 4-10 µm long ..... *Pestalotiopsis neglecta*

19(18) Conidia 5 septate, with a single apical appendage 20

20(19) Mean conidium length/width ratio > 3:1; mean conidium width 9 µm or less; mean apical appendages more than 10 µm long; conidia always 5-septate, 20.5–35.5 x 6.0–11.0 µm ..... *Seiridium eucalypti*

20(19) Mean conidium length/width ratio < 3:1; mean conidium width more than 9 µm; mean apical appendages less than 5 µm long; conidia sometimes 4–7-septate, 26.5–47.5 x 10–15 µm ..... *Seiridium papillatum*

21(17) Conidiomata setose; conidia with a single setula at both apex and base;
conidia hyaline, aseptate, fusoid, straight or slightly curved, 8–12 x 1.5–2.0 µm; setulae 5-10 µm long ..... *Dinemasporium strigosum*

21(17) Conidiomata not setose; conidia with appendages at apex only 22

22(21) Conidia bearing a funnel-shaped, extracellular appendage at the apex;
conidia hyaline, one-celled, subcylindrical to cylindrical, 15.0–22.5 x 2.5–4.0 µm ..... *Ceuthospora innumera*

22(21) Conidia bearing a rostrate or tubular, cellular appendage at the apex 23

23(22) Conidiomata pycnidial, immersed in surface of wart-like swellings of host branch tissue; conidia hyaline, one-celled, fusiform to naviculate, mostly curved, 30–45 x 3.8–5.0 µm; appendages 4.5–7.5 µm long, without septum between conidia and the appendages ..... *Ciliosporella tuberculiformis*

23(22) Conidiomata pseudostromatica, unilocular, immersed in branch tissue; conidia hyaline, one-celled, naviculate, straight or occasionally slightly curved, 15.0–21.3 x 2.5–4.0 µm; appendages 7.5–11.3 µm long, with one septum between conidia and the appendages ..... *Neoplaconema cymbiforme*

24(16) Conidia pigmented, with both transverse and longitudinal septa 25

24(16) Conidia hyaline, one-celled 27

25(24) Conidiomata acervulus; conidia large, clavate; with paraphyses; conidia with 5–8 transverse and 2–7 longitudinal susepta, brown or pale brown, 25.0–58.5 x 12.0–23.0 µm ..... *Thyrostroma eucalypti*

25(24) Conidiomata pycnidial or eustromatic; conidia small, ellipsoid; without paraphyses 26

26(25) Conidiomata pycnidial, subglobose, 400-550 µm diam; conidia 15-20 x 6.5-8.0 µm, ellipsoidal to elongate, light brown to brown, with 3 transverse and 1-2 longitudinal septa, not constricted at septa ..... *Camarosporium propinquum*

26(25) Conidiomata eustromatic mono- to multilocular, subglobose, 100-180 µm diam; conidia 10-14.0 x 8.0-10 µm, brown, muriform, with 1-3 transverse (often oblique) and 1-2 longitudinal or oblique septa, globose, subglobose, obovoide or obpyriform ..... *Dichomera eucalypti*

27(24) Conidia allantoid or straight (short cylindrical), small 28

27(24) Conidia other than allantoid in shape, obovate to ovato-rounded, large; conidia hyaline, one-celled, thick-walled, with base tapered to a distinct protuberant scar, 15.0–25.0 x 10.0–13.8 µm; microconidia one-celled, hyaline, ellipsoid, 7.0–8.5 x 3.0–4.5 µm ..... *Cryptosporiopsis* sp.

28(27) Conidia straight, short cylindrical; conidiomata dark brown; condiophores present, filiform, 20-37.5 x 1.5-2 µm; conidia 5.5-8.8 x 2-3.0 µm ..... *Phoma* sp.

28(27) Conidia curved, allantoid 29

29(28) Conidiomata subcortical, finally superficial, dark reddish brwon; conidiomata 700-1000 µm diam, 300-400 µm high; ostile single, papillate, 50-100 µm diam; conidiophores 17.5-32.5 x 1-2 µm, cylindrical, hyaline; conidia 2.5-3.5 x 1 µm ..... *Zythiostroma* sp.

29(28) Conidiomata subperidermal, immersed, dark brown or bright orange 30

30(29) Conidiomata dark brown; conidia hyaline, 3.3–5.5 x 0.8–1.2 µm .... *Cytospora eucalypticola*

30(29) Conidiomata bright orange; conidia hyaline, 2.0–6.5 x 1.0–1.5 µm .... *Endothiella gyrosa*

CHAPTER 2: PATHOGENICITY OF STEM CANKER FUNGI

2.1 INTRODUCTION

Fungal species obtained during the survey were isolated either from the margin of cankered tissue or from fruiting bodies which appeared on the lesions. As a large number of saprobic fungi can compete and survive in the necrotic tissue of a canker, it is possible that a fungus isolated from a canker may not be a primary pathogen.

Pathogenicity of tree canker fungi is generally assessed by measuring external lesions that develop after the artificial inoculation of stems (Dhingra and Sinclair 1985). In the majority of cases, artificial inoculations have been carried out on young tree seedlings grown in pots. These results may not reflect the true susceptibility of older hosts in field conditions. Many plants are susceptible to diseases only when they are very young and become resistant during the adult period (Agrios 1997). Therefore in the following experiments, both seedlings and older plantation trees were inoculated. In addition, Wardlaw (1998) has observed variations in the susceptibility of *Eucalyptus nitens* to *E. gyrosa* which appeared to be associated with bark types (smooth or rough). He reported 97% of rough barked trees developed either annual or defuse caners while only 11% of smooth-barked trees had cankers. This report could only be investigated on older trees manifesting different bark types.

Of the thirty stem fungi obtained during the survey, only a few have been previously studied as regards pathogenicity. These include *Aulographina eucalypti* (Wall and Keane 1984), *Cytospora eucalypticola* (van der Westhuizen 1965a,b; Old *et al* 1986; Shearer *et al.* 1987), *Endothia gyrosa* (Appel and Stipes 1984; Old *et al.* 1986, 1990; van der Westhuizen *et al.* 1993), *Seiridium eucalypti* (Yuan and Old 1995) and *Thyrostroma eucalypti* (Yuan 1989). However, none of these fungi or others selected for investigation (Section 2.2.1) have been tested against *Eucalyptus globulus* or *E. nitens*, the two principle plantation species in Tasmania.

2.2 MATERIALS AND METHODS

2.2.1 FUNGAL ISOLATES

Eleven fungal species were selected on the basis of collection frequency, canker severity, isolation success (availability) and reports of pathogenic behaviour in the literatures (see Section 2.4) (Table 2.2-1).

Table 2.2–1: Origin of fungal isolates used for pathogenicity tests with seedlings and trees[A]

Fungal Species	Isol. No.	Host	Locality
* *Botryosphaeria dothidea*	CF 57	*Eucalyptus stellulata*	NSW[B]
Ceuthospora innumera	ECF 55	*E. nitens*	TAS
Cytospora eucalypticola	ECF 36	*Eucalyptus* sp.	TAS
Dinemasporium strigosum	ECF 59	*E. nitens*	TAS
* *Endothia gyrosa* (TAS1)	ECF 11	*E. nitens*	TAS
* *Endothia gyrosa* (TAS3)	ECF 1	*E nitens*	TAS
* *Endothia gyrosa* (TAS9)	ECF 25	*E. nitens*	TAS
* *Harknessia* cf. *eucalypti*	ECF 51	*E. regnans*	TAS
* *Pestalotiopsis neglecta*	ECF 33	*E. nitens*	TAS
* *Phoma* sp.	ECF 54	*E. rubida*	TAS
* *Seiridium eucalypti* (1)	ECF 149	*E. regnans*	TAS
Seiridium eucalypti (2)	CF39	*E. delegatensis*	TAS
* *Seiridium papillatum*	ECF 56	*E. delegaensis*	TAS
* *Seiridium unicorne*	S1	*Callitris* sp	ACT
* *Wuestneia epispora*	ECF 57	*E nitens*	TAS
Zythistroma sp (1)	ECF 144	*E. obliqua*	TAS
Zythistroma sp (2)	ECF 147	*E. obliqua*	TAS

[A]All isolates listed in the table were used for seedling inoculation tests, but only those with asterisks were tested on old trees in the field

[B]Abbreviation ACT for Australian Capital Territory; NSW for New South Wales and TAS for Tasmania

2.2.2 INOCULUM

2.2.2.1 Mycelial inoculum

Mycelial inoculum was prepared using wheat and rice bran inoculum (Old and Kobayashi 1988). The medium was prepared with a wheat bran:rice bran: water ratio

of 1:1:2. The mixed wheat/rice bran was autoclaved in 150 ml flasks for 15 min. at 120 °C. Isolates of the tested fungi were recovered from stock cultures on PDA slopes and subcultured onto 3% MEA in darkness at 22 °C for 7 days. Four discs (ca. 1 cm^2) from the one week-old MEA cultures were then transferred into flasks with the autoclaved bran and grown for 9 days to provide inoculum for seedling and tree stem inoculations. Control inoculum was the autoclaved sterile bran.

2.2.2.2 Conidial inoculum

Pycnidia were obtained from cultures growing on 3% MEA incubated at 22 °C for 10 days under alternate cycles of 12 hours fluorescent light (36 W) and 12 hours dark. Conidial suspensions were prepared by washing the plates with sterile water. The suspensions were filtered through gauze to remove the larger pieces of agar blocks, and then 2-3 times through tissue paper. The filtered solution was centrifuged at a speed of 1000-2000 rpm to collect spores. Conidia collected were diluted with sterile water to 2 x 10^8 spores/ml. The estimation of spore concentration was carried out using a Neubauer counting chamber (hemacytometer) (Booth 1971).

2.2.3 SEEDLINGS

Potted plants of mixed provenance *E. nitens* (designated as NE-1) were bought from Woodlea Nursery, Tasmania in 15 cm plastic pots. The seeds of these plants had originated from the Australian Paper Mill (APM) forest seed orchard, Gippsland, Victoria.

The seedlings were maintained for 12 months before inoculating. At the time of inoculation, mean stem diameter of the seedlings at a height of 1 m was approx. 12 mm. All the seedlings were healthy before inoculation.

Seedlings of *E. globulus* were supplied by North Forest Products (NFP), Tasmania. After being transferred from a field nursery at NFP three month-old seedlings of the mixed provenance *E. globulus* were potted into 15-cm pots using soil fertilised with a slow release fertiliser (Osmocote). They were maintained for nine months before inoculating. At the time of inoculation, the mean stem diameter of the seedlings at a

height of 0.8 m was approximately 10 mm. All the seedlings were healthy before inoculation.

The seedlings were kept in a shade house at CSIRO, Forestry and Forest Products, Hobart. They were watered using an automatic overhead sprinkler system before and after inoculation.

2.2.4 TREES

Artificial inoculations were conducted at two *E. nitens* provenance trial sites established by Forestry Tasmania at Liffey, Deloraine District (RP 252/1) and Esperance, Geeveston District, (RP 252/2). These provenance trials were planted in the winter of 1980. Different provenances were planted in plots of 5 rows by 5 trees at Esperance and 5 rows by 6 trees at Liffey. Plots of each provenance were replicated 5 times on each site, and laid out as an incomplete Latin square design (Appendix 1 & 2). Each trial consisted of five provenances from 21 localities. At the time of inoculation, the majority of the trees were more than 25 m high with D.B.H *ca.* 25-40 cm. Stem volume, stem straightness and branch form of each provenance at age 10 were measured by Forestry Tasmania (Forestry Tasmania 1995, unpublished data).

Trees from five localities representing five provenances, designated as NE-2, NE-3, NE-4, NE-5 and NE-6 were used in the present study (Table 2.2-2).

Table 2.2-2: List of *Eucalyptus nitens* provenances used for pathogenicity tests on trees in the field[A]

No.	Provenance	Locality (Race)
* NE-2 (No.10)[B]	Errinundra	Bendoc, east Victoria
* NE-3 (No.08)	Macalister	Mt Wellington, southeast Victoria
* NE-4 (No.11)	Rubicon	Blue Range, southeast Victoria
NE-5 (No.13)	Southern New South Wales	Badja Mountain
NE-6 (No.03)	Toorongo	Powelltown, southeast Victoria

[A]All provenances listed in the table were used for cambium-deep inoculations tests at Liffey and Esperance. Only those with asterisks were used for inoculating intact or superficially wounded bark at Esperance.

[B]The numbers in the brackets are those originally designed by Forestry Tasmania for the provenance trials.

2.2.5 INOCULATION TECHNIQUES

2.2.5.1 Inoculation of seedlings

After surface-sterilising stems with 95% ethanol, a small wound was made by making a hole (3 mm diam.) to the depth of the cambium using a cork-borer. A small quantity of mycelium-mixed wheat/rice bran inoculum was placed into the wounds. Plastic film was wrapped around the wounded stem to prevent desiccation and cross-contamination of inoculated seedlings. Autoclaved fungus-free bran was used as controls. Inoculation wounds on seedlings were at 10-20 cm stem height above soil level.

2.2.5.2 Inoculation of trees

(a) Cambium-deep bark inoculation

Inoculation points on a stem were arranged in 4 columns, one at each cardinal direction. Each column consisted of 4 points 20 cm apart. The lowest point was 1 m above the ground (Fig. 2.2-1). Inoculation procedure using a cork borer was as for the seedlings.

(b) Superficial bark inoculation

Sixteen points (arranged in 4 columns *ca.* 8-10 cm apart x 4 rows 10 cm apart) were inoculated on the main stem of each tree in the experiment. The bottom row was 1 m above the ground (Fig. 2.3-13).

The bark surface to be inoculated was sterilised with 95% ethanol. A column (4 inoculation points) was either left intact before inoculation or wounded by scratching with a scalpel.

Inoculation areas around each point were defined by covering the stems with a sheet of plastic film having a small hole (1.5 cm diam.) at the centre through which inoculum was applied. Spore-suspensions were sprayed onto bark surface using a air brush (Atlantis GS 100). Mycelium-bran was applied to the bark surface using a chip of wood. The inoculated points (area) were wrapped with ducting tape.

Fig. 2.2-1: Fungal inoculations on 16-year-old smooth-barked (front) and rough-barked (rear) trees of *Eucalyptus nitens* at Esperance, south Tasmania

The inoculation points (black dots) were wrapped with plastic film.

2.2.6 EXPERIMENTAL DESIGN

2.2.6.1 Seedlings

Twelve-month-old seedlings of *E. nitens* and *E. globulus* were inoculated with the 16 isolates listed in Table 2.2-1, plus a control inoculation, to give a total of 34 treatment combinations. For each of the treatment combinations there were 5 replicates (170 seedlings in total). The 85 seedlings of each tree species were selected from a much larger group of seedlings in order to obtain trees of similar height, stem diameter and vigour.

For each tree species, the 5 replicates of each inoculation treatment were distributed into five separate blocks (85 seedlings in each). Within each block, the seedlings were randomly arranged based on a table of random numbers (Green 1968).

Inoculations for *E. nitens* were carried out in January, 1996. The first measurements of canker lesions were made two months after inoculation and then these seedlings were maintained in the shade house until seven months after inoculation when the seedlings were dissected. External lesion length, tangential spread and the length of internal discolouration were measured and reisolations were made.

The inoculations of *E. globulus* were in June 1997 (winter) and the measurements of canker lesions were made seven months after inoculation.

2.2.6.2 Trees

(a) Cambium-deep bark inoculation

Trees of five provenances, NE-2, NE-3, NE-4, NE-5 and NE-6 (Table 2.2-2) were inoculated with nine of the fungal species used in the seedling inoculation tests (Table 2.2-1). Three plots of each provenance were used (Appendix 1 & 2).

As rough and smooth-barked trees occurred in all provenance plots, paired trees of similar size in each plot (one rough- and one smooth-barked) were selected for inoculation (Fig. 2.2-1).

Each tree was inoculated with the mycelium-bran inoculum for all nine fungal species (11 isolates) and 1 control bran. Six trees (3 rough-barked and 3 smooth-barked) of each of the five provenances were inoculated giving a total of 30 trees treated per site.

The trees were inoculated in April 1996 (mid-autumn). The first measurement was made six months after inoculation and the final observations were made in February 1998 (20 months after inoculation).

Segments containing whole lesions were excised from stems of two trees (smooth- and rough-barked) of each provenance at both sites and were brought back for reisolation in the laboratory.

(b) Superficial inoculation

A successful preliminary trial with conidial-suspension and superficial inoculation techniques as described as in section 2.2.5.2 was conducted on *ca.* 25-year-old trees of *E. globulus* in late 1995 at CSIRO, Hobart. Based on this trial experiment, a field trial was designed at Esperance.

A total of 18 trees; three smooth-barked and three rough-barked trees for each of the provenance NE-2, NE-3 and NE-4, were selected for inoculation (Table 2.2-2). For each provenance, the smooth- and rough-barked trees were paired in three separate blocks. Three *E. gyrosa* isolates, TAS1, TAS3 and TAS9 (Table 2.2-1) were applied as either mycelium-bran and conidial-suspension. Each tree was inoculated following four treatments (one treatment per column):

- conidial-suspension sprayed onto intact bark;
- conidial-suspension sprayed onto bark with superficial wounds;
- mycelium-bran painted onto intact bark;
- mycelium-bran painted onto bark with superficial wounds.

The trees were inoculated in Nov. 1996 (mid-summer) and assessed in April 1997 (6 months after inoculation). The site was re-visited in November 1997 to take samples for sectioning.

2.2.7 CANKER EVALUATION

Stem canker evaluation included assessing:

1. +/- callus around or over the wound;
2. fungal sporulation in bark surrounding the inoculation point;
3. for seedlings: extent of longitudinal and tangential spread of lesions (the lesions curved tangentially around stems)
4. for trees: lesion area by the formula for the area of an ellipse (only possible for tree inoculations because of the flatter lesion on large diameter stems). Lesion area of bark was determined by the formula for the area of an ellipse:

$$A = 1/4\ L \times W \times \pi$$

where A = area of lesions; L = maximum length of lesion and W = maximum width of lesion.

5. the internal longitudinal spread of discolouration in xylem by cutting through the lesions longitudinally;
6. success of fungal reisolations.

2.2.8 REISOLATION OF FUNGUS

The presence of the fungal species in the tissues of inoculated stems was evaluated by isolation onto artificial media.

The isolations were made from the margin of discoloured tissues at inoculation points. A 20 x 5 mm longitudinal segment of stem tissue was taken from the margin of each expanding lesion. The segment was then dissected laterally into five to seven pieces. These pieces were rinsed with 95% ethanol and washed with sterile water, then surface-sterilised with 5% sodium hypochlorite for 2 min., and finally rinsed with sterile water. The surface-sterilised wood fragments were placed on 3% MEA in Petri

dish, incubated for 7-14 days at 21°C in darkness and assessed for fungal colony development.

The percentage of wood fragments each yielding the fungus inoculated was determined.

2.2.9 MICROTOME SECTIONS OF BARK

Bark segments containing lesions or healthy segments were excised from stems of smooth- and rough-barked trees. The microtome sections of the bark were prepared at the Dept of Anatomical Pathology, Royal Hobart Hospital as follows:

1. Excised bark specimens were fixed in neutral buffered formalin for several hours, followed by microwave fixation overnight at 56 °C.
2. Specimens were embedded in Fisher Tissue Prep 565 embedding compound (a mixture of purified paraffin polymers).
3. Sections (4 μm thick) were cut using a Zeiss Microm microtome and floated onto slides coated with an adhesive (aminopropyltriethoxysilane)
4. Sections were dewaxed with xylene and hydrated through decreasing concentrations of ethanol to water.
5. Sections were stained in toluidene blue for 4 min (Winsor 1994).

2.2.10 ANALYSIS OF DATA

Canker length (external and internal), tangential spread or canker area were used as the response variables for all inoculation experiments. Data were analysed using the Minitab statistical package for analyses of variance (ANOVA). Least significant difference (LSD) tests were used to test the significance of differences between treatment means.

2.3 RESULTS

2.3.1 PATHOGENICITY TO *EUCALYPTUS NITENS* AND *E. GLOBULUS* SEEDLINGS

2.3.1.1 Artificially inoculated *Eucalyptus nitens* seedlings

(a) Observations at two months after inoculation

The length of lesions and their tangential spread were significantly different ($P \leq 0.05$) to controls in the following fungi: *Phoma* sp., *S. eucalypti* (isolates 1 and 2), *E. gyrosa* (isolates TAS1, TAS3 and TAS9) and *B. dothidea* (Fig. 2.3-1; Fig. 2.3-2).

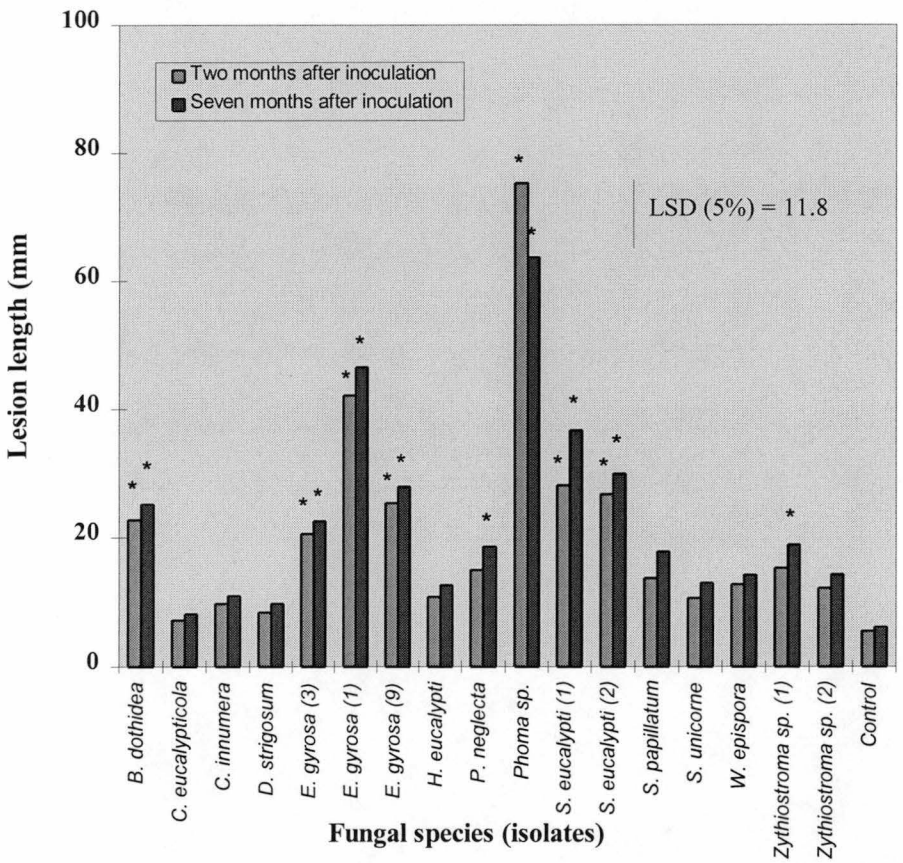


Fig. 2.3-1: Mean length (mm) of external canker lesions on stems of *Eucalyptus nitens* (NE-1) seedlings inoculated with 13 fungal species, assessed at 2 and 7 months

Bars with an asterisk are significantly different to the control

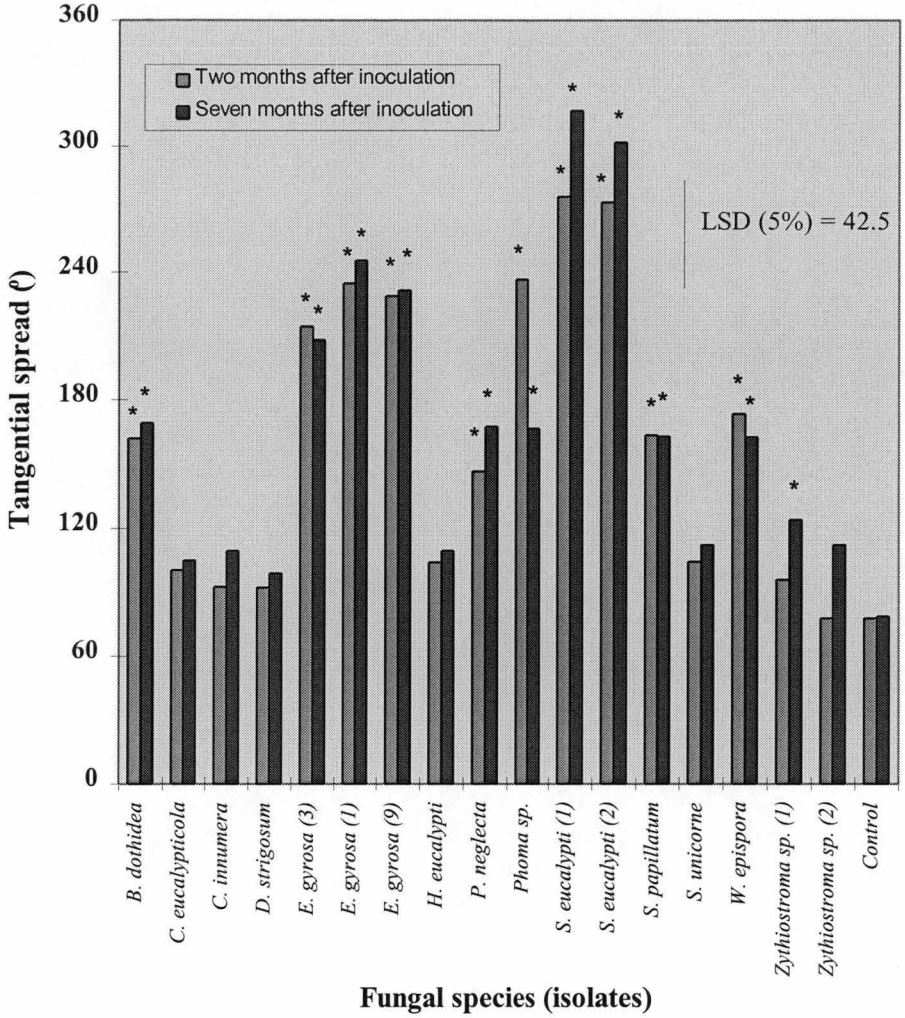


Fig. 2.3-2: Mean tangential spread of lesions on stems of *Eucalyptus nitens* (NE-1) seedlings inoculated with 13 fungal species, assessed at 2 and 7 months

Bars with an asterisk are significantly different to the control

Although *P. neglecta*, *S. papillatum* and *W. epispora* did not produce lesions which were significantly ($P \leq 0.05$) different in length to the controls, the tangential spread of the lesions caused by these fungi were significantly different to controls (Fig. 2.3-2).

Fungi which did not produce lesions that were significantly different ($P \leq 0.05$) to the controls in length or tangential spread were *C. innumera*, *C. eucalypticola*, *D. strigosum*, *H.* cf. *eucalypti*, *S. unicorne* and *Zythiostroma* sp. (isolates 1 and 2).

Of the thirteen tested fungal species, *Phoma* sp. produced the longest lesion length (Fig. 2.3-1). Dark brown to black lesions expanded rapidly in a longitudinal direction. Two months after inoculation, the maximum lesion length was up to 130 mm. Lesions were sunken or flattened with clear margins (similar to cankers observed in field), and with longitudinal cracks (Fig. 2.3-3*c*). *Phoma* sp. was the only fungus to completely girdle seedlings of *E. nitens* and cause death (Fig. 2.3-3*e*).

One isolate of *E. gyrosa* (TAS1) produced the second longest lesions. These lesions were significantly shorter than those produced by *Phoma* sp., but significantly longer than those produced by *S. eucalypti* and by the other two isolates of *E. gyrosa* (TAS3), (TAS9) (Fig. 2.3-1). Some lesions produced by this isolate nearly girdled the stems (Fig. 2.3-3*a,b*). *S. eucalypti* produced characteristic lenticular lesions in stems of all seedlings inoculated (Fig. 2.3-3*d*).

Callus development initiated within 3-4 weeks after inoculation in both the inoculated wounds and controls. All of the five wounds in the controls and the wounds inoculated with *C. innumera*, *C. eucalypticola*, *D. strigosum*, *H.* cf. *eucalypti*, *S. unicorne* and *Zythiostroma* sp (2) were completely occluded at the first assessment (Table 2.3-1). Four of the five wounds inoculated with *S. papillatum* and *Zythiostroma* sp. (1), and two inoculated with *P. neglecta* and *W. epispora* were also occluded. In contrast, no wounds inoculated with *B. dothidea*, *E. gyrosa* (all three isolates), *Phoma* sp. and *S. eucalypti* (both isolates) were occluded at this stage (Table 2.3-1).

Two months after inoculation, fungal fruiting bodies were observed on all the lesions produced by *E. gyrosa*, *H.* cf. *eucalypti*, *P. neglecta*, *Phoma* sp., *S. eucalypti*, *S. papillatum* and *Zythiostroma* sp. (Table 2.3-1). No fruiting bodies were found on any of the lesions produced by other fungi.

Table 2.3-1: *Eucalyptus nitens* (NE-1) seedlings inoculated with 13 fungal species: number of lesions occluded and with fruiting bodies present on the surface of lesions

	No. of lesions occluded		No. of lesions with fungal fruiting bodies	
Fungal species	2 months	7 months	2 months	7 months
B dothidea	0[A]	3	0[A]	5
C. eucalypticola	5	5	0	0
C. innumera	5	5	0	5
D. strigosum	5	5	0	4
E. gyrosa (TAS1)	0	0	5	5
E. gyrosa (TAS3)	0	2	5	5
E. gyrosa (TAS9)	0	2	5	5
H. cf. eucalypti	5	5	5	5
P. neglecta	2	5	5	5
Phoma sp.	0	2[B]	5	3[B]
S. eucalypti (1)	0	2	5	5
S. eucalypt (2)	0	2	5	5
S. papillatum	4	5	5	5
Seiridium unicorne	5	5	0	4
W epispora	2	5	0	5
Zythiostroma sp. (1)	4	4	5	5
Zythiostroma sp. (2)	5	5	5	5
Control	5	5	0	0

[A] Number of lesions occluded or lesions with fruiting bodies/total 5 lesions (seedlings) inoculated with each fungal species. [B]Two seedlings were dead due to inoculation of *Phoma* sp.

Table 2.3-2: Percentage of fungal reisolation from seedling stems of *Eucalyptus nitens* (NE-1) assessed seven months after inoculation with 13 fungal species

Fungal species	% Re-isolation* (mean of 5 replicates)
E. gyrosa (TAS1)	100 a
E. gyrosa (TAS3)	100 a
E. gyrosa (TAS9)	100 a
P. neglecta	100 a
C. eucalypticola	92 a
C. innumera	88 ab
Zythiostroma sp. (1)	88 ab
Zythiostroma sp. (2)	80 abc
S. eucalypt (2)	68 bcd
D strigosum	68 bcd
S. papillatum	67 cd
S. eucalypti (1)	60 cd
W. epispora	58 de
Phoma sp.	40 ef
B. dothidea	32 f
Seiridium unicorne	21 fg
H. cf. eucalypti	4 g
Control	0 g
LSD (5%)	20.9

* % Re-isolation = number of wood fragments yielding fungi inoculated/total numbers of fragments cut from lesions induced by each of the fungal isolates inoculated. Means sharing the same letter(s) are not significantly different.

(b) Observations after 7 months inoculation

External lesion size

For each fungal treatment, the lesion development pattern as observed at 7 months was very similar to that assessed 2 months after inoculation. For the parameters of lesion length and tangential spread, analysis of variance showed no significant ($P>0.05$) differences between measurements taken at 2 months and those taken at 7 months after inoculation. However, with time all the lesions had increased slightly in both length and tangential spread (Fig. 2.3-1 & Fig. 2.3-2). Of the fungal species that did not produce lesions significantly different to the controls at the first assessment, only *P. neglecta* and *Zythiostroma* sp. (1) produced significantly ($P\leq0.05$) longer lesions with greater tangential spreads than the controls at the second assessment (Fig. 2.3-1; Fig. 2.3-2).

Of the five lesions produced by *B. dothidea*, *E. gyrosa* (TAS3 and TAS9), *Phoma* sp. and *S. eucalypti* (1, 2) two or three had occluded 7 months after inoculation (Table 2.3-1). None of the lesions produced by *E. gyrosa* (TAS1) had occluded. With the exception of *C. eucalypticola*, those fungi which had not produced fruiting bodies at the first assessment had sporulating lesions by the second assessment (Table 2.3-1). Microscopic examinations showed that all three ascomyceteous fungi, *B. dothidea*, *E. gyrosa* and *W. epispora* produced anamorphs. *B. dothidea* produced the anamorph with conidiomata containing one to two-celled pigmented conidia, possibly, a species of *Botryodiplodia* or *Lasiodiplodia*. Orange-coloured fruitbodies of *Endothiella gyrosa* were abundant at the surface of all the lesions produced by inoculating with its teleomorph. *Wuestneia epispora* produced conidia of *Harknessia* cf. *eucalypti* on all lesions of inoculated seedlings.

Internal discolouration

Since internal discolouration was highly correlated ($r=0.86$, $P<0.001$ at 7 months after inoculation) with the external lesion length, data are not presented here.

Reisolation

All three isolates of *E. gyrosa* were reisolated from 100% of those wood fragments excised from the stems near the inoculated points. All other fungi producing large lesions, (*Phoma* sp., *B. dothidea* and both isolates of *S. eucalypti*) were re-isolated from 32% to 68% of the fragments, a significantly lower percentage than for *E. gyrosa* isolates (Table 2.3-2).

In contrast, those not producing significantly larger lesions than controls, such as *P. neglecta*, *C. eucalypticola*, *C. innumera*, *Zythiostroma* sp. (both isolates), *S. papillatum* and *D. strigosum* were successfully re-isolated from 67% to 100% of the fragments (Table 2.3-2).

Harknessia cf. *eucalypti* was only recovered from 4% of the fragments. However, 58% of the fragments from stems inoculated with its *W. epispora* teleomorph yielded *H.* cf. *eucalypti*.

Seiridium unicorne was re-isolated from only 21% of the fragments.

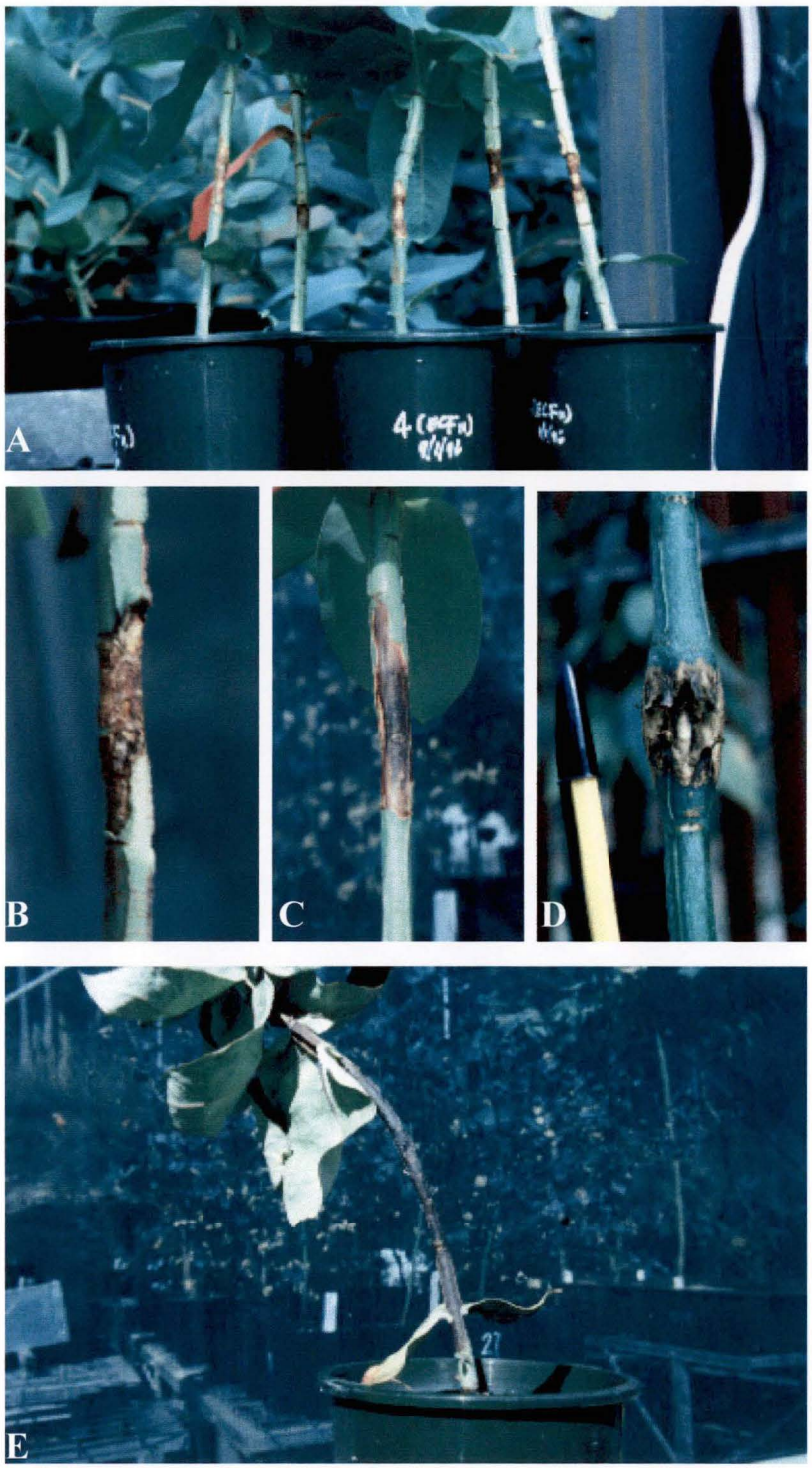


Fig. 2.3-3: Symptoms produced by stem canker fungi on 12-month-old seedlings of *Eucalyptus nitens* (NE-1) two months after inoculation

A. Seedlings inoculated with isolates of *Endothia gyrosa* (TAS1);
B. A close-up lesion of one seedling shown in A;
C. Lesion produced by *Phoma* sp.;
D. Lesion produced by *Seiridium eucalypti* (1);
E. A dying seedling inoculated with *Phoma* sp.

2.3.1.2 Artificially inoculated *Eucalyptus globulus* seedlings

Measurements of lesions for inoculations of *E. globulus* seedlings were made only 7 months after inoculation.

Similar trends to *E. nitens* (as regards lesion lengths, tangential spread and internal discolouration produced by the fungal species) were observed in *E. globulus* (Fig. 2.3-4; Fig. 2.3-5).

Botryosphaeria dothidea, *E. gyrosa* (three isolates), *Phoma* sp. and *S. eucalypti* (both isolates) produced significantly different ($P \leq 0.05$) lesion lengths and tangential extension compared to the controls.

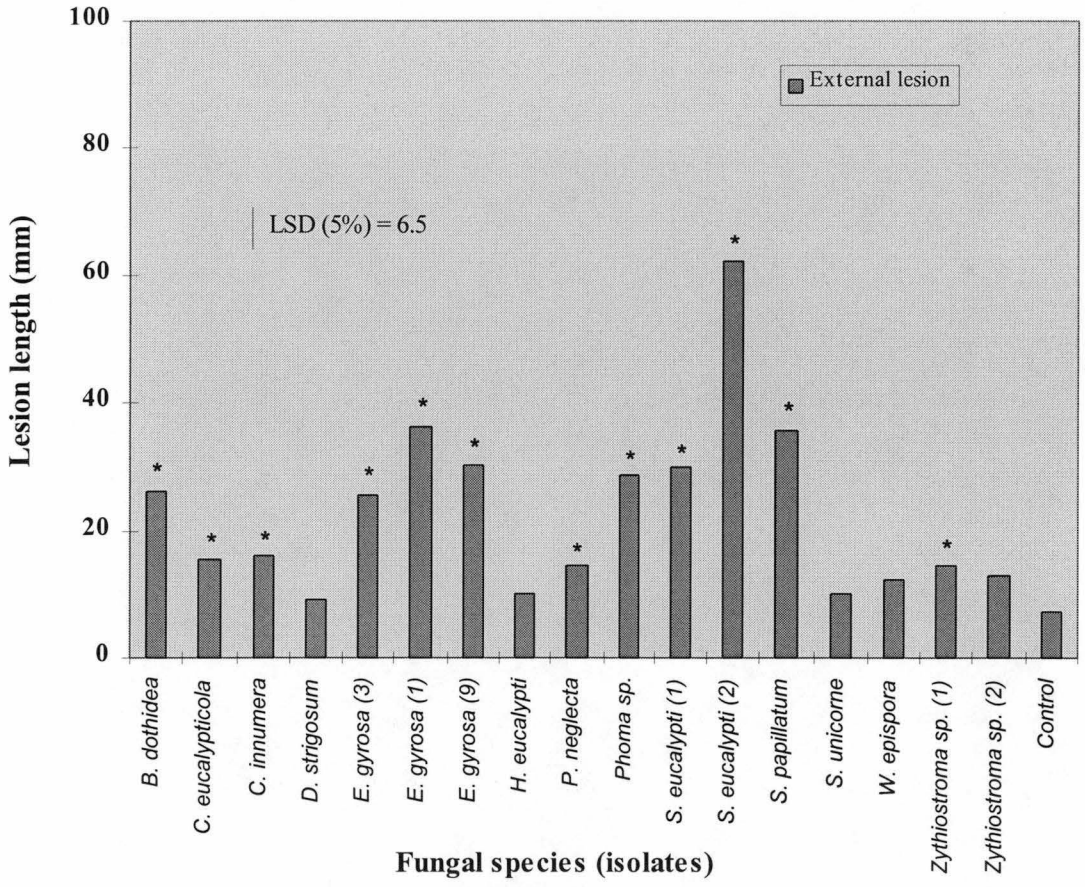


Fig. 2.3-4: Mean length (mm) of external canker lesions on stems of *Eucalyptus globulus* seedlings inoculated with 13 fungal species assessed at seven months

Bars with an asterisk are significantly different to the control

Phoma sp. appeared less aggressive and produced much shorter external lesions when inoculated on *E. globulus* as compared with *E. nitens* (Fig. 2.3-4). Lesions on this host were also significantly ($P \leq 0.05$) shorter than those produced by *S. eucalypti* (2), *E. gyrosa* (TAS1) and *S. papillatum*. Isolates of *C. eucalypticola*, *C. innumera*, *P. neglecta*, *S. papillatum* and *Zythiostroma* sp. (1) which were non pathogenic on *E. nitens* produced significantly larger lesions with greater tangential spread than the controls (Fig. 2.3-4; Fig. 2.3-5). Although *Zythiostroma* sp. (2) did not produce lesions significantly longer than the control, the tangential spread of the lesions produced by this isolate was significantly different to the control.

Compared to *E. nitens* tangential spread of fungi on *E. globulus* was much smaller 7 months after inoculation (all less than 180°, Fig. 2.3-5).

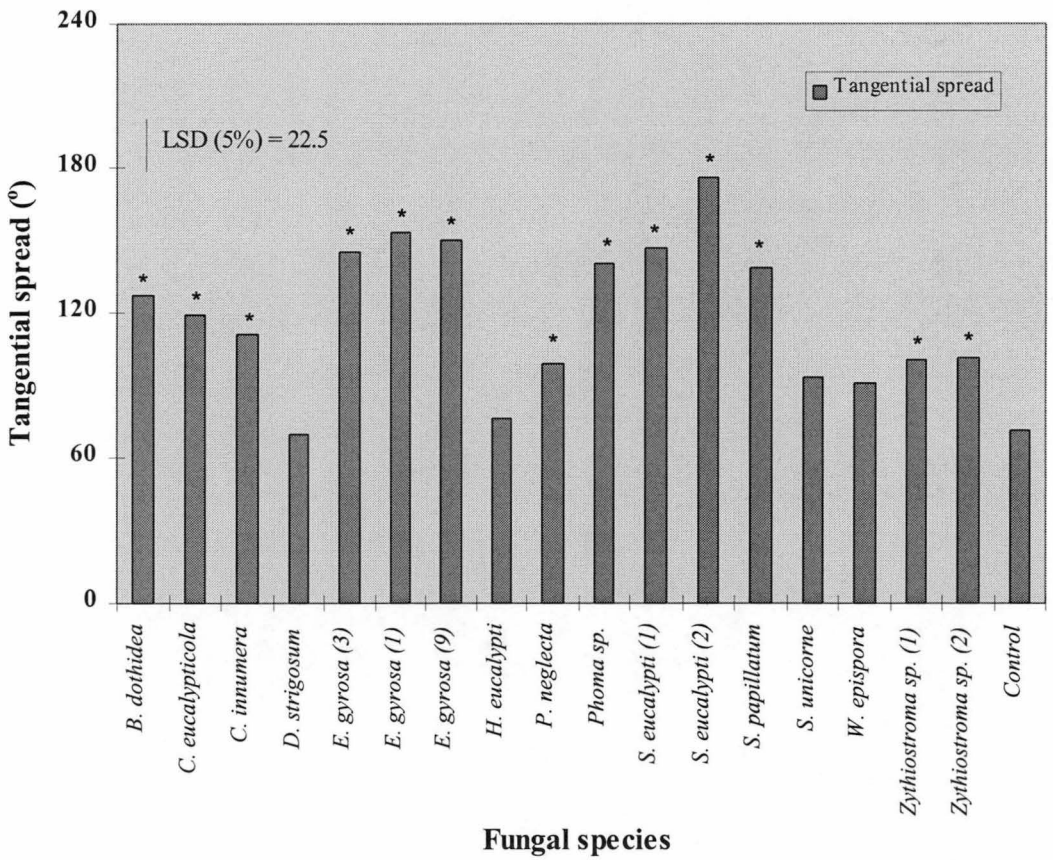


Fig. 2.3-5: Mean tangential spread of lesions on stems of *Eucalyptus globulus* seedlings inoculated with 13 fungal species assessed at seven months

Bars with an asterisk are significantly different to the control

All five lesions produced by *Phoma* sp., *E. gyrosa* (three isolates) and *S. eucalypti* (two isolates) remained open. Four lesions produced by *S. papillatum* and *Zythiostroma* sp. (1) and three produced by *B. dothidea* also remained open. All were covered with fruiting bodies 7 months after inoculation. Two lesions produced by *C. eucalypticola*, *S. unicorne* and *Zythiostroma* sp. (2) and one produced by *C. innumera* and *P. neglecta* were open. All lesions produced by *D. strigosum*, *H.* cf. *eucalypti*, *W. epispora* and the control were completely occluded. Fruiting bodies of *C. innumera*, *P. neglecta*, *H.* cf. *eucalypti*, *S. unicorne* and *Zythiostroma* sp. (2) were also found on surface of the lesions.

Internal discolouration and the external lesion length was highly correlated ($r = 0.74$, $P<0.001$, data not presented).

Reisolation of the fungi from inoculated stems showed an overall higher success than on *E. nitens*, with reisolation percentages ranging from 28% to 100% (Table 2.3-3).

Table 2.3-3: Percentage of reisolation from seedling stems of *E. globulus* inoculated with 13 fungal species assessed seven months after inoculation

Fungal species	% Re-isolation (mean of 5 replicates)
E. gyrosa (TAS1)	100 a
E gyrosa (TAS3)	100 a
E gyrosa (TAS9)	100 a
Phoma sp.	100 a
S. eucalypt (2)	96 a
Zythiostroma sp. (1)	92 a
P. neglecta	88 a
C eucalypticola	88 a
Zythiostroma sp. (2)	88 a
S papillatum	88 a
S. eucalypti (1)	84 a
C. innumera	80 a
B. dothidea	76 ab
D. strigosum	52 bc
W epispora	40 c
H. cf. eucalypti	28 c
Seiridium unicorne	28 c
Control	0 d
LSD (5%)	24.4

* % Re-isolation = number of wood fragments yielding fungi inoculated/total numbers of fragments cut from lesions induced by each of the fungal isolates inoculated. Means sharing the same letter(s) are not significantly different.

2.3.1.3 Summary of seedling artificial inoculations

An overall analysis of variance combining both data on inoculations of *E. nitens* and *E. globulus* seedlings at 7 months after inoculation revealed significant ($P \leq 0.01$) differences between the fungal species in their pathogenicity as assessed by lesion length (Appendix 3).

Based on the external lesion length and the ability to cause seedling mortality, *Phoma* sp, *S. eucalypti* and *E. gyrosa* appeared the most pathogenic to seedlings, with *S. papillatum*, *B. dothidea*, *Zythiostroma* sp. and *P. neglecta* of comparative intermediate pathogenicity and *C. innmera*, *W. epispora* (and its *H.* cf. *eucalypti* anamorph), *C. eucalypti*, *S. unicorne* and *D. strigosum* non-pathogenic (Fig. 2.3-6).

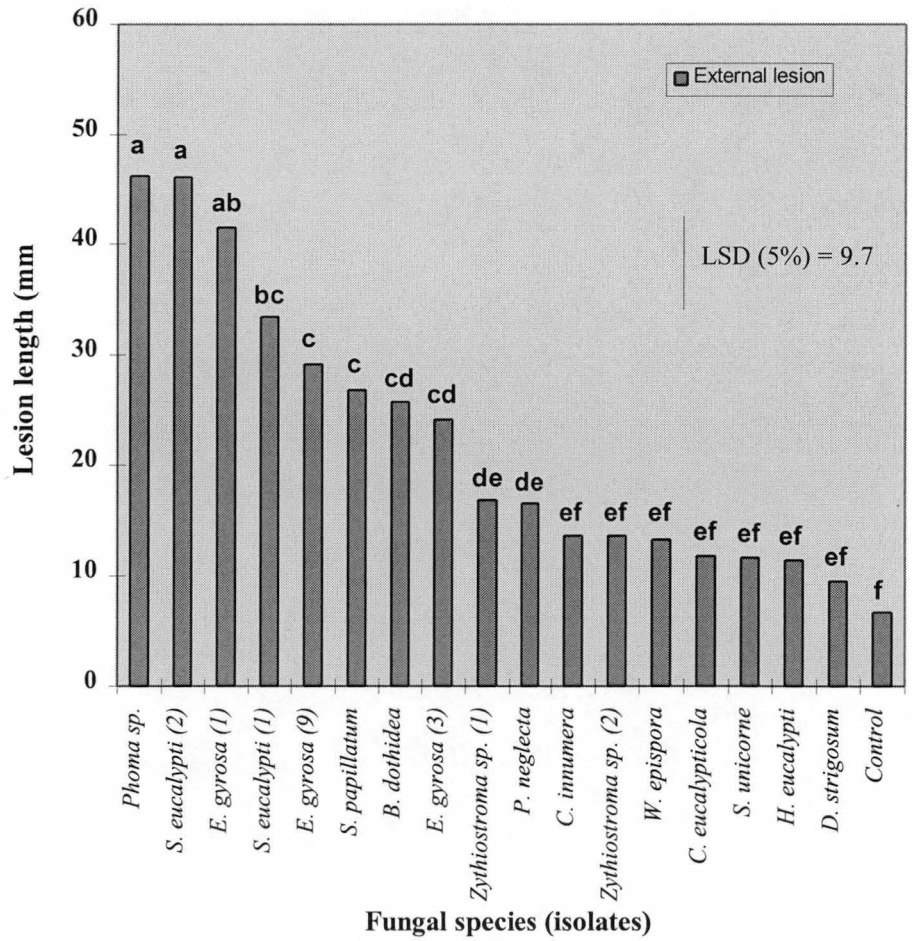


Fig. 2.3-6: Combining mean length (mm) of external canker lesions on stems of *Eucalyptus nitens* and *E. globulus* seedlings inoculated with 13 fungal species seven months after inoculation

Bars sharing the same letter(s) are not significantly different

When comparing the two eucalypt species, significant interaction differences ($P<0.05$) between fungi and host species can be observed (Appendix 3).

The two *Seiridium* species, *S. eucalypti* (2) and *S. papillatum* produced significantly larger lesions on *E. globulus* than on *E. nitens* (Fig. 2.3-7).

The lesions of *Phoma* sp on *E. nitens* were significantly longer than those on *E. globulus*.

In general, those fungi less aggressive to *E. nitens*, such as *C. eucalypticola* and *C. innumera* appeared more pathogenic to *E. globulus*.

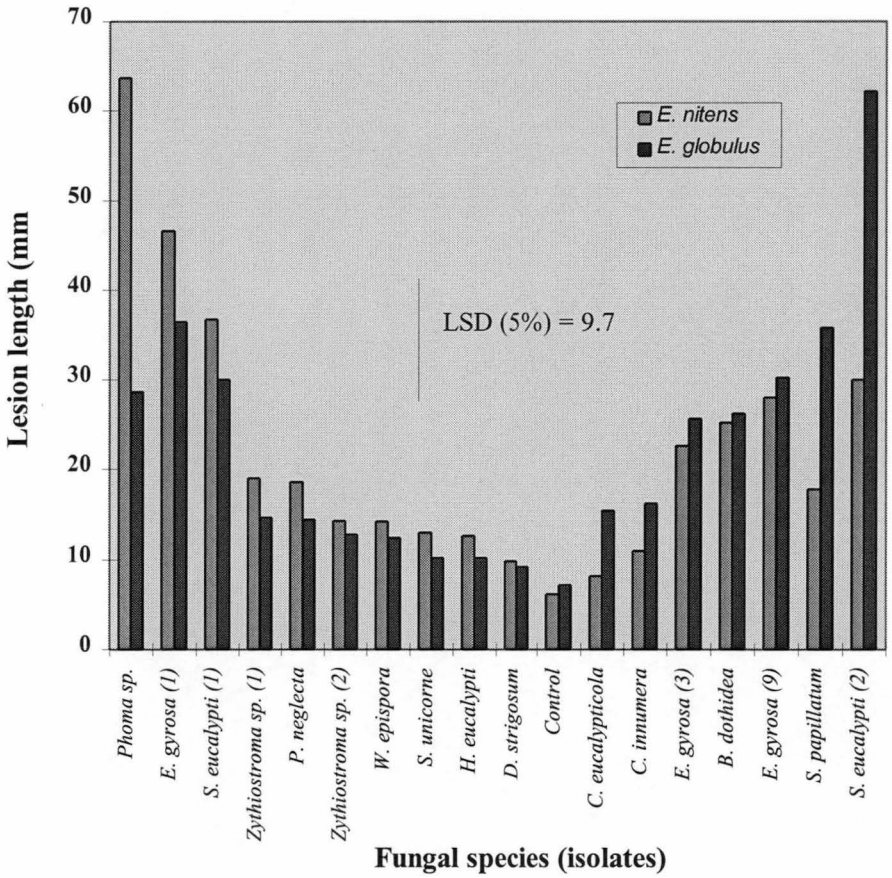


Fig. 2.3-7: Mean length (mm) of external canker lesions on *Eucalyptus nitens* and *E. globulus* seedlings inoculated with 13 fungal species, seven months after inoculation

2.3.2 PATHOGENICITY TO 16 YEAR-OLD *EUCALYPTUS NITENS*

An overall ANOVA shows there are significant differences ($P \leq 0.05$) between fungi, provenances, plantation sites, bark types and assessment times in external lesion size (area) assessed 6 and 20 months after inoculation (Appendix 3).

2.3.2.1 External lesion size

(a) Observations at 6 months

Only *Phoma* sp., *E. gyrosa* (TAS1) and *E. gyrosa* (TAS9) produced lesions significantly ($P \leq 0.05$) different to those in stems receiving control inoculations (Fig. 2.3-8). Other fungal species or isolates, *S. eucalypti* (1), *E. gyrosa* (TAS3), *B. dothidea*, *S. papillatum*, *P. neglecta*, *H.* cf. *eucalypti*, *W. epispora* and *S. unicorne* did not produce lesions significantly different to those of the controls.

Endothia gyrosa (TAS1) produced the largest lesions, followed by *Phoma* sp. and *E. gyrosa* (TAS9). Lesions produced by *E. gyrosa* (TAS1) were significantly ($P \leq 0.05$) larger than those of *E. gyrosa* (TAS9), but not significantly larger than those produced by *Phoma* sp. (Fig. 2.3-8).

(b) Observations after 20 months

Lesions increased significantly ($P \leq 0.05$) in size between 6 and 20 months after inoculation.

Similar to observations 6 months after inoculation, *E. gyrosa* (TAS1) produced the largest lesions, followed by *Phoma* sp. and *E. gyrosa* (TAS9) (Fig. 2.3-8).

Of the fungal species that had not produced lesions significantly different to controls at the first assessment, only *S. eucalypti* (1) and *E. gyrosa* (TAS3) had now significantly ($P \leq 0.05$) larger lesions compared with controls.

Botryosphaeria dothidea, S. papillatum, *P. neglecta H.* cf. *eucalypti*, *W. epispora* and *S. unicorne* did not produce lesions significantly different to controls (Fig. 2.3-8).

The lesions produced by the fungal species on 16-year-old trees of *E. nitens* in plantations were morphologically similar to those on seedlings in the shade house. However, infections by all fungal species did not produce perennial or diffuse cankers. Lesions were restricted to bark tissue and had mostly occluded by 20 months. No necrosis was observed in sapwood 20 months after inoculation (Fig. 2.3-9*a,b*).

In common with the seedling tests, relatively low reisolation percentages of *Seiridium unicorne* (12.5%), *H.* cf. *eucalypti* (45%) and *W. epispora* (as *H.* cf. *eucalypti*) (42.8%) were obtained from discoloured tissue around the margins of lesions. All other tested fungal species were recovered at a high percentage of reisolation: 80% for *B. dothidea*, 85.7% for *P. neglecta* and 100% for *E. gyrosa*, *Phoma* sp., *S. eucalypti* and *S. papillatum*.

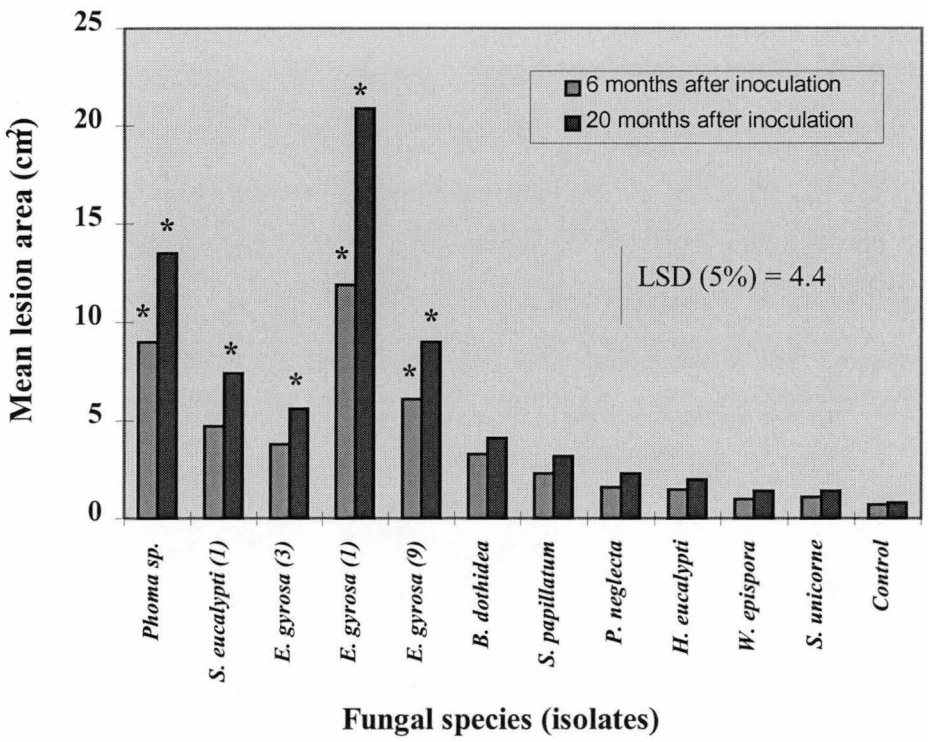
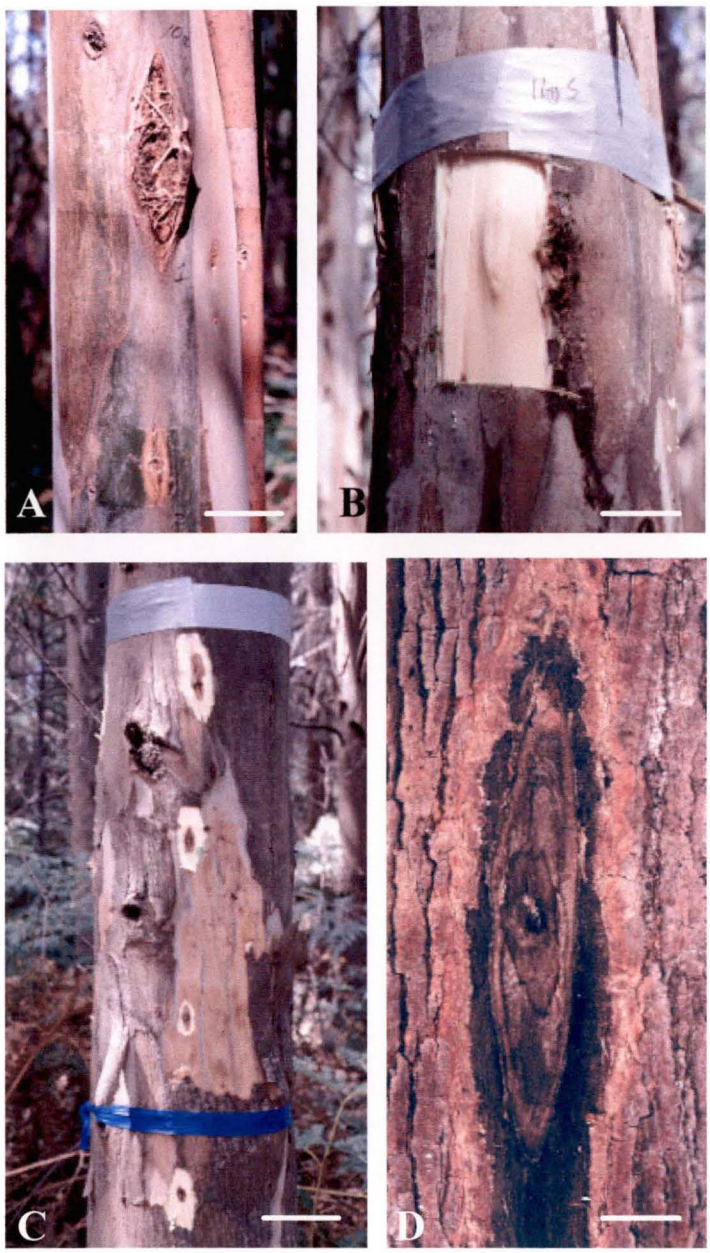


Fig. 2.3-8: Overall mean lesion area (cm^2) on 16-year-old *Eucalyptus nitens* stems inoculated with 9 fungal species 6 and 20 months after inoculation

Each bar represents mean area from 60 lesions (3 replications x 5 provenances x 2 bark types x 2 sites). Bars with an asterisk are significantly different to controls.



Bar = 50 mm for A; 70 mm for B; 90 mm for C and 7 mm for D.

Fig. 2.3-9: Symptoms produced by *Endothia gyrosa* on 16-year-old trees of *Eucalyptus nitens*

A. Provenance NE-2; upper lesion produced by TAS1, lower lesion produced by TAS9;
B. Provenance NE-4; lesion produced by TAS1 has been removed (note absence of internal discolouration);
C. Provenance NE-3; top three lesions produced by isolates TAS1, TAS9, TAS3, control inoculation at bottom of picture;
D. Provenance NE-3 (rough barked); a lesion produced by TAS1

2.3.2.2 Site

Differences in lesion size were found between sites. Lesions in trees at Esperance site tended to be larger than those in trees at Liffey (Fig. 2.3-10).

As expected, significant differences between the two sites ($P \leq 0.01$) could only be observed for those fungi (*E. gyrosa*, *Phoma* sp. and *S. eucalypti*) which caused lesions significantly larger than control inoculations.

Six months after inoculation with *E. gyrosa* (TAS1), the mean lesion area (including all five provenances) at Esperance was significantly larger than at Liffey (Fig.2.3-10a). At Esperance *Phoma* sp. produced a significantly larger mean lesion area compared to Liffey only on a single provenance (NE-6).

As lesion size increased with time, differences between the two sites became more pronounced.

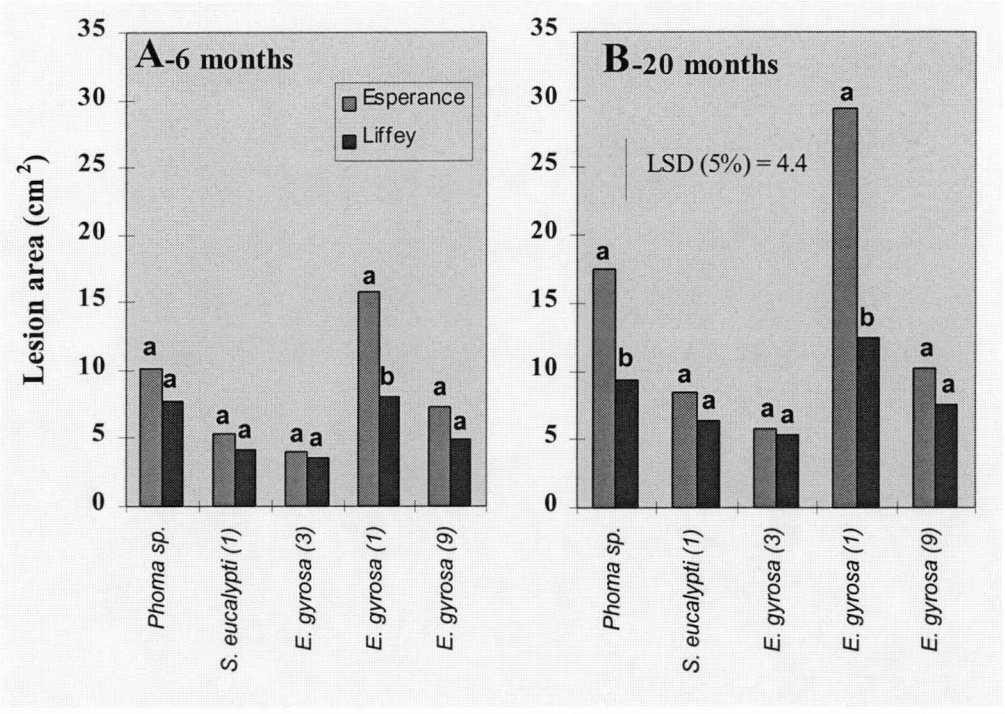


Fig. 2.3-10: Overall mean lesion areas produced by five fungal isolates on trees of 16-year-old *Eucalyptus nitens* at two sites (Esperance and Liffey) assessed 6 and 20 months after inoculation

Each bar represents mean lesion area from 30 trees (3 replications x 5 provenances x 2 bark types). Bars sharing the same letter within each fungal isolates are not significantly different

Twenty months after inoculation with both *E. gyrosa* (TAS1) and *Phoma* sp., mean lesion area (including all five provenances) was significantly larger at Esperance than at Liffey (Fig. 2.3-10b). At 20 months, the lesions produced by *S. eucalypti* (1) on NE-6 and *E. gyrosa* (TAS9) on two provenances (NE-4 and NE-5) at Esperance were also significantly larger than those at Liffey. However, overall means for all five provenances for *S. eucalypti* (1) and *E. gyrosa* (TAS9) were not significantly different between the two sites.

2.3.2.3 Provenances

There were significant differences ($P \leq 0.05$) in lesion size between the five provenances. This was only found at 20 months for lesions produced by *E. gyrosa* (TAS1), *Phoma* sp. and *S. eucalypti* (1) (Table 2.3-4).

Table. 2.3-4: Comparison of the mean lesion area (cm^2) produced by three fungal species between five provenances of 16-year-old *Eucalyptus nitens* assessed 6 and 20 months after inoculation[A]

		Fungal species					Mean
Time	Provenance	*E. gyrosa* (3)	*E. gyrosa* (1)	*E. gyrosa* (9)	*Phoma* sp.	*S eucalypti* (1)	of row
	NE-2	3.6	13.5	6.0	8.5	5.4	**7.4a**
6	NE-3	3.0	10.7	5.0	9.2	4.1	**6.4a**
m	NE-4	4.3	13.5	6.7	8.1	4.3	**7.4a**
	NE-5	4.2	10.9	6.7	8.5	4.1	**6.9a**
	NE-6	3.8	11.2	6.2	10.7	5.7	**7.5a**
	NE-2	5.2	19.0b	7.3	**10.5c**	7.4b	**9.9b**
20	NE-3	5.0	17.0b	7.3	**15.0ab**	6.2b	**10.1ab**
m	NE-4	6.0	**25.5a**	11.0	**11.2bc**	7.3b	**12.2ab**
	NE-5	5.9	19.3b	9.5	13.7abc	**11.9a**	**12.1ab**
	NE-6	5.9	**23.7a**	10.0	**17.0a**	**15.4a**	**14.4a**

[A]Values are the mean lesion area (cm^2) of 12 lesions (3 replicates x 2 bark types x 2 sites).
[B]Mean lesion area between bark types within each provenance with different letters are significantly different (LSD 5% = 4.4).

Endothia gyrosa (TAS1) produced the largest lesions on NE-4, a Rubicon provenance from southeast Victoria, while *Phoma* sp. and *S. eucalypti* (1) produced the largest on NE-6, a Toorongo provenance also from southeast Victoria (Table 2.3-4). Overall,

NE-6 appeared to be the most susceptible to the infection and NE-2, a Errinundra provenance from east Victoria was the least susceptible (Table 2.3-4).

On the basis of overall mean lesion area produced by the fungal isolates listed in Table 2.3-3, the order of provenance susceptibility from high to low assessed at 20 months is as follows:

NE-6 >NE-4 > NE-5 > NE-3 >NE-2

2.3.2.4 Artificial inoculation of different bark types

(a) Cambium-deep inoculation with 9 fungal species

Lesions on smooth-barked trees tended to be larger than those on rough-barked trees (Fig. 2.3-11). However, these differences (over the mean for all five provenances) were only significant ($P \leq 0.05$) at 20 months for *Phoma* sp. and *E. gyrosa* (TAS1) (Fig. 2.3-11b).

An ANOVA showed that provenances, fungal isolates and time significantly affected lesions size on different bark types (Appendix 3).

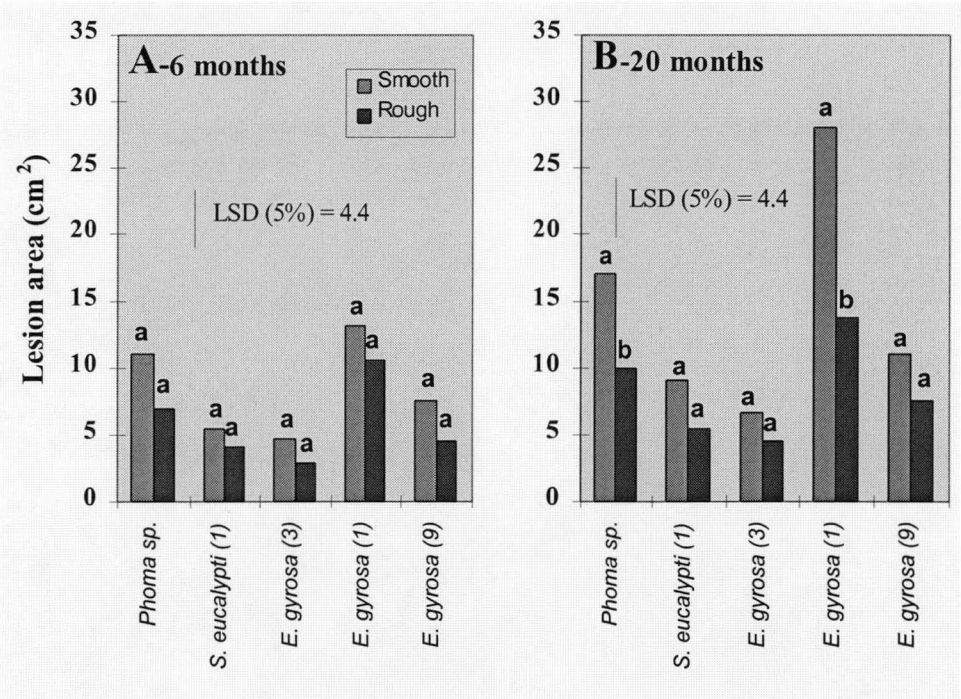
Significant differences ($P \leq 0.05$) in lesion size were observed at six months between the smooth- and rough-barked inoculations only with more aggressive fungal isolates, *E. gyrosa* (TAS1), (TAS9) and *Phoma* sp. for provenances NE-2, NE-3, NE-5 and NE-6 (Table 2.3-5).

Twenty months after inoculation significant differences between smooth- and rough-barked trees in lesion size were found within all provenances inoculated with *E. gyrosa* (TAS1) and *Phoma* sp. (Table 2.3-5). At this assessment, the less aggressive isolates, *E. gyrosa* (TAS3) and *E. gyrosa* (TAS9) on NE-4 and *S. eucalypti* (1) on NE-6 also produced significantly larger lesions on smooth-barked trees than those on rough-barked trees (Table 2.3-5). Nearly all isolates produced significantly larger

lesions on smooth-barked trees than rough-barked trees for NE-4 and NE-6, the two most susceptible provenances.

With time, the differences in lesion size within provenances were greatly increased, but varied with provenances. For example, the difference in lesion size between smooth and rough barked trees of NE-6, the most susceptible provenance had increased from 1.9 cm^2 at 6 months to 23.9 cm^2 at 20 months, while for NE-2, the most resistant provenance, increased only from 4.5 cm^2 to 11.8 cm^2 (Table 2.3-5).

Fig. 2.3-11: Overall mean lesion areas produced by five fungal isolates on smooth- and rough-barked trees of 16-year-old *Eucalyptus nitens* assessed 6 and 20 months after inoculation



Each bar represents mean lesion area from 30 trees (3 replications x 5 provenances x 2 sites).
Bars sharing the same letter within each fungal isolates are not significantly different

Table. 2.3-5. Comparison of lesion area (cm^2) for bark types (smooth and rough) of 16-year-old *Eucalyptus nitens* inoculated with five stem fungi assessed 6 and 20 months after inoculation[A]

			Fungal species				
Time	Provenance	Bark	*E gyrosa* (3)	*E. gyrosa* (1)	*E. gyrosa* (9)	*Phoma* sp.	*S. eucalypti* (1)
6 m	NE-2	Smooth	4.3	**15.7a**	7.1	10.1	6.1
		Rough	2.9	**11.2b**	4.9	6.9	4.7
	NE-3	Smooth	3.9	12.1	6.8	**13.2a**	4.8
		Rough	2.1	9.2	3.2	**5.2b**	3.4
	NE-4	Smooth	6.1	14.7	8.2	8.5	5.0
		Rough	2.4	12.3	5.2	7.6	3.6
	NE-5	Smooth	4.0	11.3	7.7	**10.7a**	4.2
		Rough	4.3	10.5	5.6	**6.3b**	3.9
	NE-6	Smooth	5.0	12.1	**8.4a**	12.6	6.8
		Rough	2.6	10.2	**4.0b**	8.8	4.5
20 m	NE-2	Smooth	6.0	**24.9a**	8.9	**12.9a**	8.9
		Rough	4.4	**13.1b**	5.9	**8.0b**	5.9
	NE-3	Smooth	5.3	**21.0a**	8.1	**21.0a**	8.0
		Rough	4.7	**12.9b**	6.4	**8.9b**	4.4
	NE-4	Smooth	**8.3a**	**31.9a**	**13.6a**	**13.5a**	8.8
		Rough	**3.7b**	**19.0b**	**8.3b**	**8.9b**	5.7
	NE-5	Smooth	6.0	**26.4a**	11.6	**16.7a**	7.2
		Rough	5.7	**12.3b**	7.4	**10.6b**	4.7
	NE-6	Smooth	7.5	**35.6a**	**12.6a**	**20.3a**	**12.2a**
		Rough	4.2	**11.7b**	**7.3b**	**13.7b**	**6.7b**

[A]Values are the mean lesion area (cm^2) of 6 trees (3 replicates x 2 sites).
[B]Mean lesion area between bark types within each provenance with different letters are significantly different (LSD 5% = 4.4).

(b) Superficial inoculation of bark with E. gyrosa

Intact bark

Six months after inoculation, no lesions or fruiting bodies of *E. gyrosa* were observed at/around the inoculation points for both smooth- and rough-barked trees when intact bark was inoculated with conidial-suspensions. However, at a single conidial-suspension inoculation point on one rough-barked tree of NE-3 a lesion had developed and fruiting bodies of *E. gyrosa* (TAS3) were found one year after inoculation (Fig. 2.3-13*b*).

No lesions or fruitbodies of *E. gyrosa* were produced for intact bark inoculation of smooth-barked trees when inoculated with mycelium-bran. On 2 out of total 27 inoculation points for rough-barked trees (each of NE-2 and NE-3) fruiting bodies of

E. gyrosa (TAS1) were present and lesions had developed. No lesions or fruiting bodies were observed on other inoculation points when examined 12 months after inoculation.

With scratched wounds

When bark was scratched prior to inoculation, all the inoculation points (whatever the provenances, isolates and inoculum types) formed lesions and produced fruiting bodies of *E. gyrosa* (Fig. 2.3-13*a*) on both smooth and rough-barked trees.

External lesions produced by both conidial and mycelial inoculum on smooth bark were more or less ellipsoidal in shape. Six months after inoculation the maximum lesions produced by mycelial and conidial inoculation were 53.0 cm^2 on NE-4 (smooth-barked) and 13.7 cm^2 on NE-2 (smooth-barked) respectively. All the lesions on smooth-barked trees produced by both conidia and mycelium were significantly ($P \leq 0.05$) larger than those of controls (Fig. 2.3-12).

On rough-barked trees, although no significant differences in lesion lengths were found between the lesions of any inoculation and controls (Fig. 2.3-12), fungal infections were observed in all the inoculated bark, presenting conidiomata of *E. gyrosa* around wounding points.

Inoculum type (conidia vs mycelium), fungal isolates and bark type of trees signficantly ($P \leq 0.01$) affected lesion size. In common with the cambium-deep bark inoculations, lesions on rough-barked trees superficially inoculated were again much smaller than those on smooth-barked trees (Fig. 2.3-12).

Thin sections through both healthy and infected bark of rough and smooth-barked *E. nitens* showed the same differences in bark structure between rough and smooth bark. As shown in Fig. 2.3-14 and Fig. 2.3-15, the cork layers of the rough bark are much thicker compared with the smooth bark. The cortex cells below the cork layers in rough bark are smaller, thick-walled and arranged closely (Fig. 2.3-15*a,c*), while in smooth bark, they are large, thin-walled and loosely arranged (Fig. 2.3-14*c*). In

addition, medullary rays are more disorganised in the rough bark (Fig. 2.3-15*b*) than those in the smooth bark (Fig. 2.3-14*b*).

The sections through infected smooth and rough bark also showed that the conidiomata of the fungus appeared to be limited within the bark layer, mainly in cork layers (Fig. 2.3-14*a*) or in cortex (Fig. 2.3-15*a*).

The development of cankers within the bark layer was apparently affected by the differences in the above-mentioned anatomical characteristics of bark type.

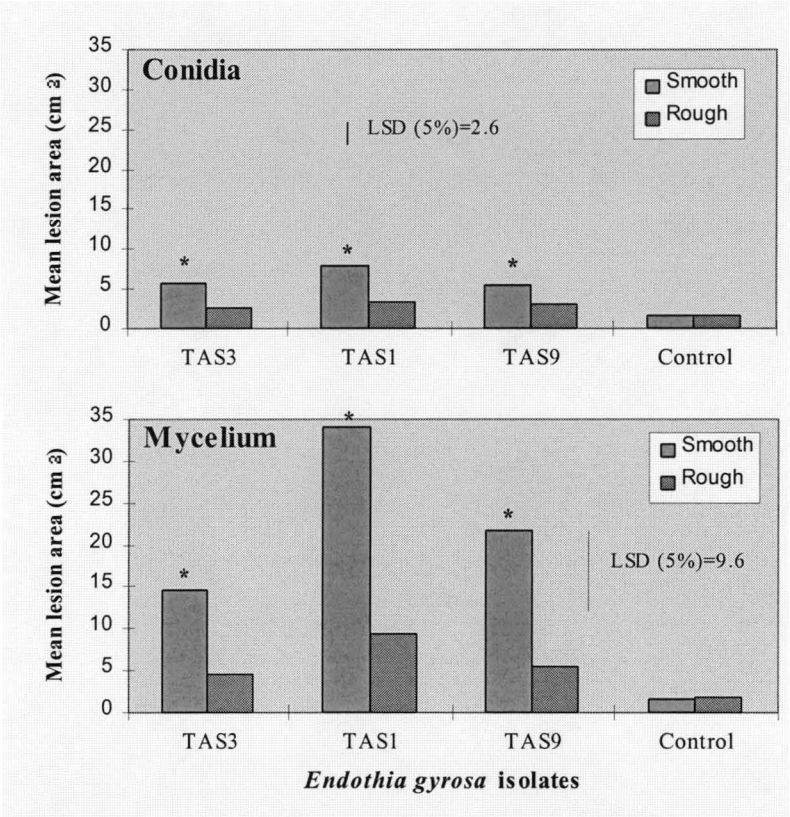


Fig. 2.3-12: Mean lesion area (cm^2) in smooth- and rough-barked stems of three *E. nitens* provenances inoculated with conidia and mycelium of *Endothia gyrosa* isolates through scratched wounds on stem surface

Each bar represents mean area from 9 lesions. Bars with an asterisk are significantly different from controls at a particular LSD value

Fig. 2.3-13: Inoculation of three *Endothia gyrosa* isolates onto the intact and superficially wounded bark of smooth- (A) and rough- (B) barked trees of 16-year-old *Eucalyptus nitens*

A. Nos. 5-7: intact bark inoculated with mycelium;
Nos. 9-11: scratched wounds inoculated with conidia;
Nos. 13-15: scratched wounds inoculated with mycelium;
Nos. 4, 8, 12 and 16: controls

B. Rough-barked tree (NE-3); intact bark inoculated with conidia of TAS3, note irregular-shaped lesion and conidiomata (black dots)

Fig. 2.3-14: Light micrographs of transverse sections through smooth bark of *Eucalyptus nitens*

A. Conidioma (c) of *Endothia gyrosa* immersed in cork layers of smooth bark (conidial inoculation into scrached wound);
B. Healthy smooth outer bark showing more organised medullary rays (mr);
C. Healthy smooth outer bark showing thin cork layers (cl) and irregularly and loosely arranged large, thin-walled cortex cells (cc) below the cork layers

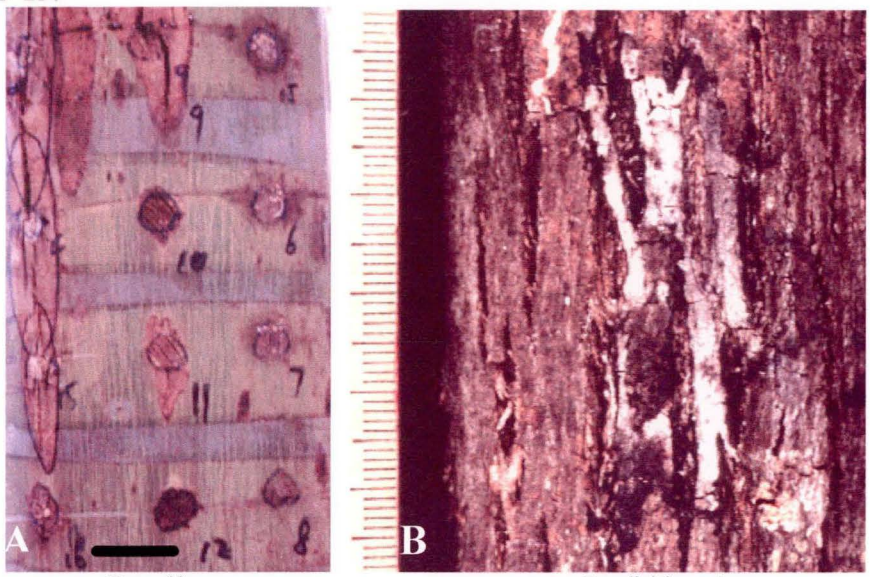
All sections were stained with Toluidine blue

Fig. 2.3-15: Light micrographs of transverse sections through rough bark of *Eucalyptus nitens*

A. Conidioma (c) of *Endothia gyrosa* deeply immersed in cortex of rough bark (mycelial inoculation into scratched wound);
B. Healthy rough outer bark showing disorganised medullary rays (mr);
C. Healthy rough bark showing thick cork layers (cl) and closely arranged small, thick-walled cortex cells (cc) below the cork layers

All sections were stained with Toluidine blue

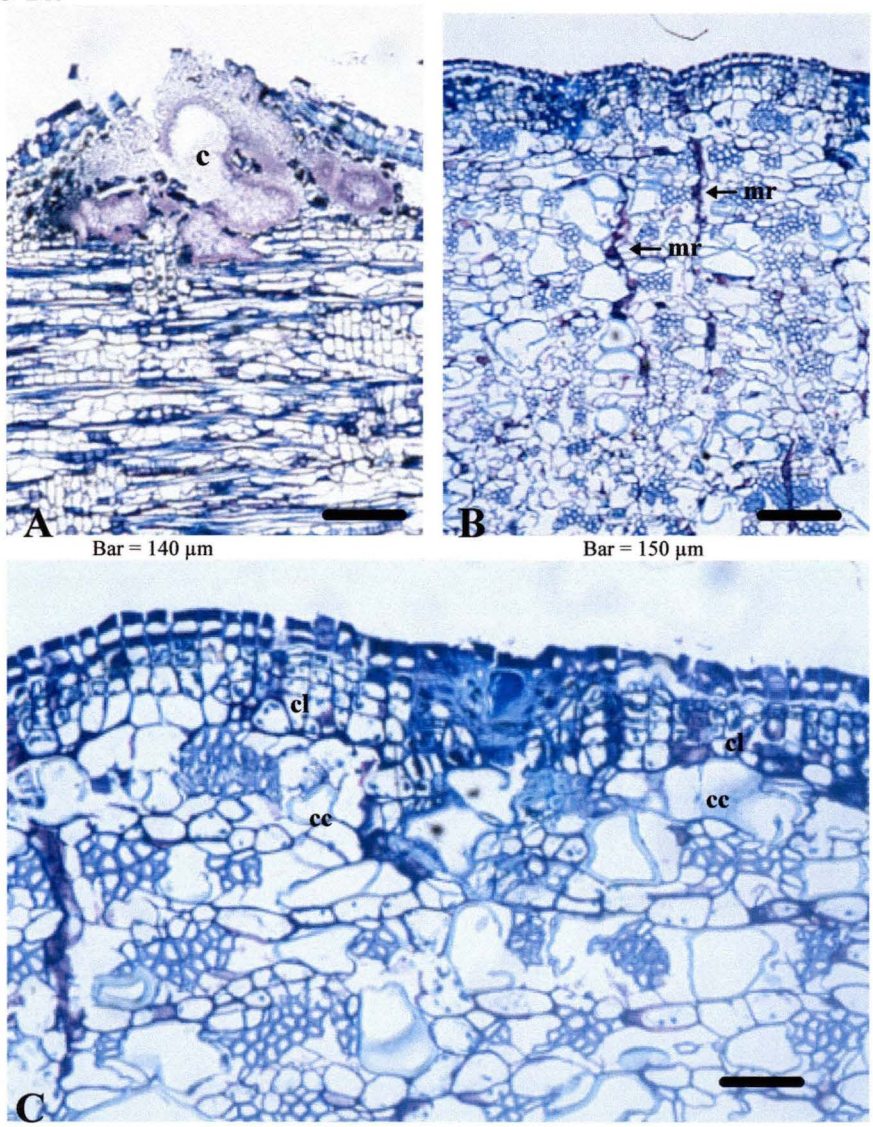
Fig. 2.3-13:



Bar = 10 cm

One division = 1 mm

Fig. 2.3-14:



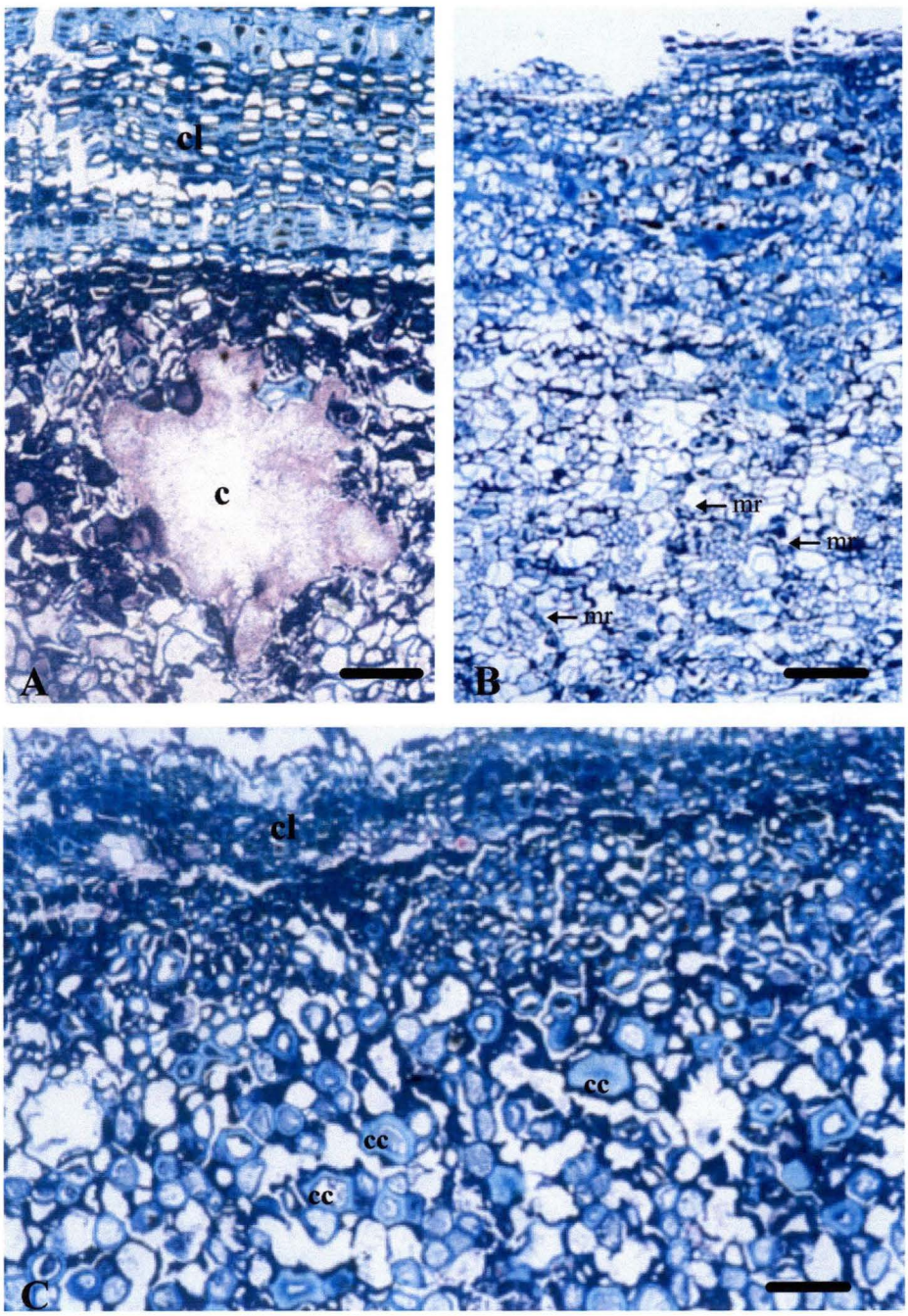
Bar = 140 µm

Bar = 150 µm

Bar = 55 µm

Fig. 2.3-15:

Bar = 80 µm for **A** 65 µm for **B**



Bar = 40 µm for **C**

2.4 DISCUSSION

PATHOGENICITY OF TESTED FUNGAL SPECIES

The fungal species tested in the study could be classified into three groups based on their ability to cause stem canker lesions following artificial inoculation on both seedlings and trees of *Eucalyptus nitens* and *E. globulus*:

1. pathogenic species; always producing comparatively large cankers on both seedlings and trees of the tested hosts; *Phoma* sp., *Endothia gyrosa* and *Seiridium eucalypti*;
2. intermediate or weakly pathogenic species; artificial inoculation occasionally caused lesions larger than control wounds, especially on *E. globulus*; *Botryosphaeria dothidea*, *Seiridium papillatum*, *Pestalotiopsis neglecta*, *Zythiostroma* sp., *Ceuthospora innumera* and *Cytospora eucalypticola*;
3. non pathogenic species which do not produce cankers under the experimental conditions tested; *Dinemasporium strigosum*, *Seiridium unicorne*, *Harknessia* cf. *eucalypti* and its *Wuestneia epispora* teleomorph.

Phoma sp was investigated because of its association with a large, sunken canker on the main stem of *Eucalyptus rubida* Dean & Maiden in the field, although it was encountered only once during the present survey (Chapter 1). Pathogenicity of *Phoma* is also supported by reports of other species of the genus as eucalypt stem canker agents eg. *P. eucalyptica* Sacc. (Azevedo 1971; Gibson 1975). Hence the ability of the *Phoma* species to cause large sunken canker lesions and seedling mortality was not unexpected. In view of this demonstrated pathogenicity, further investigation of its occurrence on *E. rubida* and other eucalypt species is needed. Although the morphological and cultural characteristics of this fungus were examined in this study (Yuan, Chapter 1) the fungus cannot be formally described until more cankered material is found.

Pathogenicity of *E. gyrosa* and *S. eucalypti* has been previously studied in various inoculation tests on other eucalypt species (Old *et al* 1986, 1990; Shearer *et al.* 1987; van der Westhuizen *et al.* 1993; Yuan and Old 1995). They were included in the present tests because of their comparatively high frequency of collection and association with stem cankers. The pathogenicity of *E. gyrosa* and *S. eucalypti* to *E. nitens* and *E. globulus* (demonstrated for the first time in this study) confirms previous reports of their pathogenicity to eucalypts (Old *et al.* 1986, 1990; Shearer *et al.* 1987; van der Westhuizen *et al.* 1993; Yuan and Old 1995).

A *Botryosphaeria* species, *B. obtusa* (Schw.) Shoemaker with *Sphaeropsis* anamorph has been recorded on *Crataegus*, *Malus* and *Pyrus* in Tasmania (Sampson and Walker 1982). *Botryosphaeria dothidea* has yet to be found in Tasmania but was included in the investigation because it is a recognised pathogen associated with stem cankers of eucalypts in mainland Australia (Davison and Tay 1983; Shearer *et al.* 1987; Old *et al.* 1990), the state of Florida, USA (Barnard *et al.* 1987) and South Africa (Smith *et al.* 1994).

It is surprising that isolate (CF57) of *B. dothidea* tested in this study did not produce significantly larger cankers than the controls on 16-year-old trees of *E. nitens* in the field inoculations. Only small or moderate sized cankers were induced in seedlings of *E. nitens* and *E. globulus* in the shade house.

Davison and Tay (1983) reported that *B. dothidea* (as *B. ribis*) produced large cankers on coppice stems of *E. marginata* Donn ex Smith in pathogenicity tests in Western Australia. Fraser and Davison (1986) also found that inoculation of 13-year-old plantation trees of *E. saligna* Smith with *B. dothidea* caused the largest cankers among the three tested fungi including *E. gyrosa* (as *Endothiella*) and *C. eucalypticola*. In South Africa, pathogenicity of *B. dothidea* was tested on 2-year-old trees of *E. nitens* and caused cankers with lengths ranging 32-64 mm 3 months after inoculation (Smith *et al.* 1994).

Very weak pathogenicity of *B. dothidea* as observed by the author might be explained by questioning the identity of the *B. dothidea* isolate used for artificial inoculation. This isolate (CF57) of *B. dothidea* was isolated from a stem canker on *E. stellulata* Sieber ex DC with crown dieback in New England (NSW) and was identified as *B. ribis* (= *B. dothidea*) by Yuan (1989) from teleomorph and colony morphology. The presence of the pigmented *Botryodiplodia* (Sacc.) Sacc. or *Lasiodiplodia* Ellis & Everh. anamorph in the lesions on stems inoculated with isolate CF57 suggested that the present fungus was not *B. dothidea*. Neither *B. dothidea* nor *B. ribis* produce pigmented conidia, but hyaline conidia belonging to the genus *Dothiorella* Sacc. (Sivanesan 1984).

Pestalotiopsis neglecta was tested on account of its reported pathogenic ability to cause blight or spots on leaves and shoots of *E. globulus* and *E. grandis* in India (Sharma *et al.* 1985). The Tasmanian isolate of *P. neglecta* behaved as a weak canker pathogen.

This fungus does remain viable in the lesion tissue as indicated by a high level of successful reisolations. *P. neglecta* has also been isolated from several healthy bark samples of plantation *E. nitens* in Tasmania (Yuan unpublished data). In a wounding experiment in Orbost, Victoria, Old *et al.* (1993) often recovered *Pestalotiopsis* from the sapwood of wounded *Eucalyptus sieberi* trees. They also isolated *Pestaloptiopsis* from the seedling stems of several *Eucalyptus* inoculated with other stem fungi (*Chalara eucalypti* Z.Q. Yuan & Kile, *Diaporthe eucalypticola* Old & Z.Q. Yuan and *E. gyrosa*). Two species of the genus *Pestalotiopsis* have been reported as endophytic on eucalypts. *P. versicolor* (Speg.) Stey. was found present in healthy twigs of mature *E. nitens* in Canberra (Fisher *et al.* 1993). More recently *P. guepini* (Desm.) Stey. was isolated from healthy twigs of *E. grandis* in Uruguay (Bettucci and Alonso 1997). *P. neglecta* is possibly another endophytic *Pestalotiopsis* of eucalypts.

Seiridium papillatum, the newly described species on eucalypts (Yuan & Mohammed 1997b) and *Seiridium unicorne* (Cooke & Ellis) Sutton isolated from *Callitris* sp. in

the Australian Capital Territory (ACT) were included in the tests to be compared with *S. eucalypti*.

Unlike *S. eucalypti* which has consistently proved pathogenic to eucalypts in artificial inoculations, *S. papillatum* did not show a similar pathogenic ability to *E. nitens* and *E. globulus*, although it did produce significantly longer lesions than controls on seedlings of *E. globulus* and on smooth-barked trees of *E. nitens* in field inoculations. This weak pathogenicity agrees with field observations. The fungus was only collected from dead branches.

Seiridium unicorne has been reported as a primary canker pathogen on cypress under natural conditions (Boyce and Graves 1966; Swart 1973; Sasaki and Kobayashi 1975; Boesewinkel 1983; Xenopoulos 1991). In artificial inoculation tests, *S. unicorne* was reported as causing cankers on wounded shoots of *Chamaecyparis lawsoniana* and *Cupressus sempervirens* (Spanos *et al.* 1996). The present isolate of *S. unicorne* from *Callitris* sp. showed no ability to cause canker lesions on eucalypts, either seedlings or trees. The pathogenicities of the three *Seiridium* species tested in the study supports their separation based on other attributes such as morphology and DNA polymorphism (see Chapter 1).

Zythiostroma sp was tested because of its frequent collection on cankered stems and the strong pathogenic ability demonstrated by a *Zythiostroma* species isolated from cankers on *Banksia coccinea* R. Brown in Western Australia (Shearer *et al.*, 1995). The present *Zythiostroma* species tested by artificial inoculation was, in the field, associated with several stem cankers on dead branches (Chapter 1) but proved to be only weakly pathogenic to *E. globulus* and not pathogenic to *E. nitens*.

Among the weakly pathogenic fungi, *C. eucalypticola* is the only species that has been previously studied in artificial inoculations. This fungus was found commonly associated with the dead branches of stressed trees (Chapter 1). In common with the isolates studied in other states of Australia (Davison and Tay 1983; Old *et al.* 1986), the isolate of *C. eucalypticola* isolated from eucalypts in Tasmania proved to be only

weakly pathogenic. However on *E. globulus* seedlings, lesions were significantly larger than those of controls. This fungus was described as a canker pathogen of young *E. saligna* in South Africa (van der Westhuizen 1965b).

The present isolate of *C. innumera* was collected from dead branches of *E. nitens*. Prior to this study it was only recorded in Australia as a saprobic or leaf spotting fungus on the leaves of several eucalypts (Sankaran *et al.* 1995). *C. innumera* was originally described on dead eucalypt leaves from Tasmania in 1899 (Saccardo and Sydow 1902) and has been recorded on leaves of several eucalypts in Australia as a saprobic or leaf spotting fungus (Sankaran, Sutton and Minter 1995). The fungus showed no pathogenicity to seedlings of *E. nitens* but was weakly pathogenic to *E. globulus* seedlings, although several *Ceuthospora* species have been reported to be associated with cankers and dieback of conifers in Canada (Funk 1981).

Dinemasporium strigosum was included in the test because it was found in the survey (Chapter 1) for the first time on dead woody stems, though it has been recorded on leaves of *Acacia* in Australia (Yuan 1996). This isolate of *D. strigosum* did not show any pathogenicity to seedlings of both *E. nitens* and *E. globulus*. This is consistent with earlier reports of the fungus as a common saprophyte on Gramineae (Sutton, 1980).

Harknessia cf. *eucalypti* along with its teleomorph *W. epispora* did not produce lesions significantly different to the controls on any of the two eucalypt species tested. Low reisolation percentage from the tissue around inoculation points indicated it had failed to establish well in living host tissue. The pathogenicity of this fungus was tested because it was associated with severe cankers in an *E. regnans* plantation (Chapter 1), although mainly on senescent branches or dead suppressed branches in the lower part of the crown. Most of the other known species of *Harknessia* do not appear to be aggressive parasites. *H. eucalypti* has been found on leaves or occasionally small dead twigs (Sutton 1980; Nag Raj 1993). Swart (1972) observed that both *Harknessia renispora* H.J. Swart on *Melaleuca squarrosa* and

H. uromycoides on *Platylobium obtusangulum* were found only on leaves already colonised by *Seimatosporium* spp.

HOST PATHOGEN INTERACTIONS

While it is generally accepted that canker length is a measure of virulence (Griffin *et al* 1984) pathogenicity testing in tree species is made difficult by the complexity of host-pathogen inter-relationships which influence canker length, sapwood invasion and callus formation. These inter-relationships as shown in this study are influenced by host species, bark type, provenance, age and length of time an infection has been established. Site can exert a strong influence on host response in field trials.

Influence of host species on pathogenicity

Seedlings of *E. globulus* appeared to be slightly less resistant to fungal infection than those of *E. nitens*. Some fungal species, *P. neglecta*, *S. papillatum*, *Zythiostroma* sp, *C. innumera* and *C. eucalypticola* only produced significantly larger lesions than controls on seedlings of *E. globulus*. Lesions at 7 months on *E. globulus* had a lower occlusion rate than those on *E. nitens*. Transplant shock from the field nursery to pots could have been a significant factor explaining the greater susceptibility of *E. globulus*. However plants had been maintained for nearly a year before inoculating and were healthy at the time of inoculation. Comparative levels of pathogenicity for the different fungal species or isolates on *E. globulus* are not entirely consistent with those expressed on *E. nitens*. For example, *Phoma* sp produced the longest lesions on *E. nitens*, but not on *E. globulus*. This differential host response may indicate that the increased susceptibility of *E. globulus* was not simply a reflection of stress related susceptibility.

Research has already shown that, in artificial inoculation tests, certain eucalypt species are more susceptible to stem canker fungi than others. Yuan (1989) tested five species, *B. dothidea*, *C. eucalypticola*, *E. gyrosa*, *S. eucalypti* and *Thyrostroma*

eucalypti on 13-month-old seedlings of 9 eucalypt species in the glass house. Of the tested eucalypts, *E. cypellocarpa* L. Johnson was the most susceptible species to *B. dothidea*, *E. gyrosa* and *S. eucalypti*. Old *et al.* (1993) also found that four out of five fungal isolates tested (*E. gyrosa*, *Endothiella* sp., *Diaporthe* sp. and *Phomopsis* sp.) produced the longest lesions on 12-month-old seedlings of *E. cypellocarpa*. The lesions were significantly longer than those produced by the same fungal isolates on other eucalypt species, such as *E. obliqua* 2 months after inoculation. Seedlings of both *E. obliqua* and *E. sieberi*, were the most resistant to *E. gyrosa*, in two separate pathogenicity tests carried out by Old *et al.* (1993) and Yuan (1989).

It is difficult to detect any clear pattern in previous assessments of susceptibility or resistance to stem canker fungi. *E. cypellocarpa* is one of the southern blue gums in the series *Globulinae*, along with *E. nitens* and *E. globulus* belonging to the subgenus *Symphyomyrtus* (Eldridge *et al.* 1993). *E. obliqua* and *E. sieberi* both belong to the subgenus *Monocalyptus*. Eucalypt species in the subgenus *Monocalyptus* may be more resistant to canker fungi than those in *Symphyomyrtus*. However Old *et al.* (1990) reported that *E. regnans* and *E. delegatensis* (both in the subgenus *Monocalyptus*) were less resistant to canker fungi than *E. grandis* and *E. saligna* of the subgenus *Symphyomyrtus*, although these interspecific differences were not as marked as those between, for example, *E. cypellocarpa* and *E. obliqua*.

Influence of host vigour on pathogenicity

The greater susceptibility of *E. nitens* at the Esperance plantation in comparison to Liffey may be due to poorer growth at Esperance which is a poorer site (Forestry Tasmania, internal report).

The data assessed at age 10 in 1990 by Forestry Tasmania indicated lower stem volumes at Esperance for all provenances. For example, stem volumes for provenances NE-2, NE-3, NE-5 and NE-6 were, respectively, 94.8 (m^3ha^{-1}), 172.9, 181.9 and 120.8 at Liffey, while 59.4, 111.9, 114.4 and 94.8 at Esperance.

Woody plants are generally predisposed to canker diseases when the host trees are under stress (Schoeneweiss 1975). Studies have shown that the susceptibility and severity of canker diseases associated with *E. gyrosa* and *B. dothidea* are often related to environmental stress, such as drought stress and defoliation by insects (Crist and Schoeneweiss 1975; Hunter and Stipes 1978; Appel and Stipes 1984; Old *et al.* 1990, Smith *et al.* 1994). Hunter and Stipes (1978) observed that infection of pin oak by *E. gyrosa* was greater during the driest months of a year. Appel and Stipes (1984) proved that the colonisation by *E. gyrosa* was only successful when the inoculated pin oak trees were under water stress. Old *et al* (1990) found seedlings of eucalypts subjected to water stress were not predisposed to canker formation by *E. gyrosa* and *B. dothidea* but did admit to problems in creating valid experimental conditions. However, when the trees were defoliated, either manually or by severe insect attack, canker development was significantly increased (Old *et al.* 1990).

Field observations often report cankers associated with evident stress in trees. During the present survey, for example, *C. eucalypticola* was observed on 30-year-old trees of *E. globulus* and *E. pulchella* stressed by fire damage. Perithecia of *E. gyrosa* were abundant on slash and remaining living trees in a 5-year-old plantation of *E. nitens* heavily defoliated by autumn gum moth (Chapter 1). In northern Tasmania, *S. eucalypti* was found several times at one single locality where an *E. nitens* plantation was also severely defoliated by *Mycosphaerella tasmaniensis* Crous & M.J. Wingf. (Old and de Little pers. comm.).

Influence of bark type on pathogenicity

The present study revealed a difference in canker development between *E. nitens* trees with either smooth or rough bark type in the lower stem.

In a study on stem cankers of *E. nitens* associated with *E. gyrosa* in Tasmania, Wardlaw (1998) noted a clear association of the disease with rough-barked trees. He reported that 97% of rough-barked trees developed either annual or diffuse cankers while only 11% of smooth-barked trees had cankers. He suggested that the difference

could be due to the presence of longitudinal cracks in rough-barked trees which provide suitable infection courts. It was very unlikely that rough bark formation was a response to infection since healthy rough-barked trees could be found at other sites in Tasmania. Both smooth and rough-barked individual trees occur in all *E. nitens* provenances (Chippendale 1988).

Association of tree bark type with susceptibility to disease infection has been noticed on other tree species. Boyce (1933, 1961) found cankers on Douglas fir in northern California caused by *Phomoposis lokoyae* Hahn occurred on both main stems and branches with relatively smooth bark. Gotwols *et al.* (1980) reported thick-barked trees of *Fagus grandifolia* Ehrh. were more resistant to canker formation by *Nectria coccinea* var. *faginata* Lohm. Watson & Ayers than thin-barked trees.

In the present study, it has been clearly shown that infection of *E. gyrosa* and other fungal species following artificial inoculation, spread significantly faster in smooth bark with much larger external lesions compared with that in rough bark. The apparent anomaly between observations of naturally infected trees by Wardlaw (1998) where rough-barked trees were most susceptible, and this result in which wound-inoculated smooth-barked trees were more susceptible may be explained by the different anatomical features illustrated in Fig. 2.3-14 & Fig. 2.3-15 and the fact that infection was artificial.

Sections through rough and smooth bark clearly revealed that, in rough bark, the arrangment of dense cork layers and small, thick-walled, closely arranged cortex cells below these layers could provide a physical barrier, limiting or at least delaying canker formation. In comparison and as suggested by Wardlaw (1998) the more organised anatomical arrangement of the medullary rays in smooth barked *E. nitens* could facilitate the spread of growing hyphae in the bark. Such an explanation has been invoked for invasion of *Picea abies* (L.) Karst bark by *Heterobasidion annosum* (Fr.) Bref. (Lindeberg and Johansson 1991).

The results obtained with artificial inoculations support Wardlaw's hypothesis that the longitudinal cracks on bark surfaces of rough-barked trees may provide suitable infection courts for *E. gyrosa* which is a wound fungus. On trees with intact bark none of the inoculations with sprayed conidia or painted mycelium on smooth-barked trees were successful. With rough barked trees, one inoculation with conidia and two with mycelium produced lesions and conidiomata of the inoculated fungus. Inoculated cork-borer wounds or scratched bark wounds (exposing inner bark tissues) all became infected (resulting lesions were more extensive in smooth barked trees).

The apparently higher susceptibility of rough barked trees in comparison to smooth barked trees can be explained by differences in bark anatomy and not physiological or chemical responses to infection. Once the outer bark is breached smooth-barked trees of *E. nitens* are actually more susceptible to fungal infection than rough-barked trees due to the anatomical structure of smooth-barked trees facilitating post-penetration fungal invasion.

Thinning at early stage to remove rough-barked trees may be a practical exercise to protect trees from stem canker disease. However, under natural conditions, trees often receive various wounds due to insect, wind and hail damage which expose the inner bark to invasion by wound fungi. Since smooth-barked trees are more susceptible in the post penetration phase, this may be a poor option.

Influence of provenance on pathogenicity

Among the inoculated provenances of *E. nitens* in the field, whatever the bark type and at both sites in the trials, two southeast Victorian provenances appeared more susceptible to fungal infection (ie NE-6, a Toorongo provenance from Powelltown and NE-4, a Rubicon provenance from Blue Range). An Errinundra provenance from Bendoc (NE-2), east Victoria was consistently the more resistant.

Although there appeared to be differences in provenance resistance to canker fungi it is unlikely this is a factor which will be considered in current Australian breeding and

selection programs for *E. nitens* or *E. globulus*. Field inoculations in this study and others (Old *et al.* 1986, 1990) indicate that these canker diseases do not pose a sufficient threat to warrant the additional cost of resistance screening.

In other countries where the use of clonal eucalypts in forestry is current practice and the threat of canker diseases more serious resistance selection is important. In Brazil planting resistant clones of eucalypts is the only control method for *Cryphonectria cubensis* (Alfenas *et al.* 1997). In South Africa, screening of eucalypt clones for tolerance of *E. gyrosa* cankers is currently in progress (van der Westhuizen *et al.* 1993).

Influence of host age and period of time after artificial infection

Most fungi in this study caused more severe lesions on 12-month-old seedlings of *E. nitens* than on 16-year-old trees. Fungal infection penetrated the sapwood of seedlings although only in one case caused death 2 months after inoculation. Infections on tree stems were comparatively superficial. No perennial or diffuse cankers developed as a result of artificial inoculation. Most of the cankers including those caused by comparatively pathogenic fungi (eg. *E. gyrosa*, *Phoma* sp. and *S. eucalypti*) were eventually occluded and confined to the bark 20 months after inoculation. These variations in the response of seedling and trees to the same pathogens can be attributed to marked anatomical and physiological differences between seedlings and mature trees (Akai and Fukutomi 1980).

Comparisons between field trials investigating the pathogenicity of eucalypt canker fungi are difficult, often because of the discrepancies in host age. Yuan (1989) found that *E. gyrosa* and *B. dothidea* (as *B. ribis*) did clearly cause more severe damage in field trials than this study. Sapwood was attacked and internal discolouration evident only 3 months after artificial inoculation. However, the trees inoculated were younger (3 years) and the species inoculated different (*E. delegatensis*, *E. grandis* and *E. regnans*). The trees had also been stressed before inoculation by artificial defoliation. In more comparable field trials to the present study with 12 year old

E. sieberi, stained sapwood was associated with small *Endothiella* cankers (40 mm) six months after inoculation but this discolouration was quite restricted (Old *et al.* 1993).

The results of Old *et al* (1993) highlighted the importance of the length of time after artificial inoculation in the expression of pathogenicity. Some large cankers observed by Old *et al.* at 10 weeks on 12 year old trees did not evolve with time and remained superficial. Other cankers continued development doubling in size by the time of the next assessment at six months.

Canker on seedlings in this study developed rapidly within 2 months following inoculation but then extension ceased with very little difference between observations at 2 months and 7 months after inoculation. Canker development on trees was much slower. It is only at 20 months that clear differences in pathogenicity, bark type, site and provenances could be detected. Although canker development, sapwood invasion and callus formation with time was different for trees and seedlings fungal pathogenicity patterns on seedlings were consistent to those on trees. Unless specific information is required in relation to pathogenicity (ie. the influence of adult bark type on canker development) seedling inoculation techniques prove worthwhile providing a quick (within 2 months) assessment for pathogenicity under controlled and more easily experimentally comparable conditions.

VIABILITY OF FUNGI

Viability of stem inhabiting fungi in living tissue may be of some importance to the incidence of canker diseases. As observed in this study, fungal species able to cause significant lesions were more easily re-isolated from tissue around lesions than were non-pathogenic fungi. *E. gyrosa*, for example, was always isolated from 100% of either open or occluded lesions, indicating persistence in both living and dead tissue around inoculation wounds. During the survey this fungus was also isolated from the healthy bark of both smooth and rough barked *E. nitens* at Tewkesbury, northern Tasmania (Yuan unpublished data).

Fungi such as *E. gyrosa* may be well adapted to saprophytic behaviour in dead tissue and also occur as latent symptomless endophytes in bark tissue. *Botryosphaeria dothidea* which is associated with die-back and canker diseases of various *Eucalyptus* species in South Africa was found to be the dominant taxon occurring as an endophyte in healthy leaves of *E. grandis* and *E. nitens* (Smith *et al.* 1994). It is suggested that with *B. dothidea* disease symptoms may result from the manifestation of previous latent infections after the onset of stress.

Stem disease symptoms could possibly result opportunistically both from latent infections and saprophytic growth on dead tissue. The role of slash in the inoculum build-up of such fungi must be given careful thought when developing management prescriptions.

POTENTIAL IMPACT OF CANKER FUNGI ON EUCALYPT FORESTS

Canker fungi have not been reported as a significant problem in either natural forests or eucalypt plantations in any parts of Australia, except for the outbreak of *E. gyrosa* canker in *E. nitens* plantation observed in 1993 by Wardlaw (1998) and recent reports of damage to 3 year old *E. globulus* in Western Australia (Shedley, pers. comm.).

Pathogenicity studies with eleven fungal species from the survey proved that majority of the fungal species were weak pathogens or saprophytes, not causing severe canker lesions on trees. Even though they appeared to be aggressive on seedlings when inoculated artificially, the limited penetration of these fungal infections to bark tissue on the large, healthy trees may explain why cankers are usually rare in field conditions. It is suggested that the fungal species tested in the present study are probably opportunists and only cause significant cankers when the hosts are stressed due to other predisposed factors such as drought, defoliation and suppression.

However *E. gyrosa* is common across southeastern Australia and Western Australia. Considering its performance in pathogenicity tests, damage caused by this fungus to vigorous eucalypts in Tasmania (Wardlaw 1998) and plantations overseas (van der

Westhuizen *et al.* 1993) it is potentially the most threatening fungal species to the eucalypt forest industry. In South Africa this fungus is considered as a serious pathogen on clones of various eucalypt species and has caused severe damage to timber (Brits and Grey 1992, unpublished booklet).

With the fast changing silvicultural environment is it just a question of time before *E. gyrosa* or another canker fungi yet to be discovered can make a devastating impact? *Cryphonectria cubensis*, for example, the most devastating canker pathogen of plantation eucalypts in many parts of the world (Boerboom and Maas 1971; Hodges *et al.* 1976, 1979; Sharma *et al.* 1985; Wingfield *et al.* 1989; Conradie *et al.* 1990) has been isolated from root cankers of *E. marginata* in Western Australia (Davision and Coates 1991).

CHAPTER 3: ENDOTHIA GYROSA: A DETAILED INVESTIGATION

3.1 INTRODUCTION

Endothia gyrosa was one of the most frequently encountered fungal species during the survey (Yuan and Mohammed 1997a). In the survey cankers of *E. gyrosa* were often observed on trees which appeared stressed ie. with symptoms of crown dieback, defoliated or suppressed. Along with its *Endothiella* anamorph *E. gyrosa* was ubiquitous throughout Tasmania on many eucalypt species in plantations and natural forests. In mainland Australia it has also been recorded in association with stem cankers on a wide range of eucalypt species (Appendix 4).

In the previously described pathogenicity tests (Chapter 2) *E. gyrosa* proved comparatively pathogenic in artificial inoculations of canker fungi to both seedlings and trees. Previous studies in Australia have concluded that *E. gyrosa* is opportunistic (Old *et al.* 1986, 1990). However, it is currently causing significant problems in both Western Australia (Shedley 1998, pers. comm.) and South Africa (van der Westhuizen *et al.* 1993). It impacted severely on the growth of a vigorous 16-year-old *E. nitens* plantation in Tasmania, with resulting mortalities (Wardlaw 1998). These reports initiated an in-depth investigation of variation in pathogenicity, cultural characteristics (ie morphology, fungicide response), vegetative compatibility and DNA polymorphism among both Australian and overseas isolates of *E. gyrosa*.

DNA polymorphism

Walker *et al.* (1985) compared American and Portuguese herbarium materials of *E. gyrosa* with Australian specimens and concluded that the latter were conspecific with American and Portuguese *E. gyrosa*. Presumably *E. gyrosa* represents a single species with a world wide distribution (Roane *et al.* 1974; Barr 1978; Walker *et al.* 1985), although never investigated.

The origin of *E. gyrosa* in Australia remains unknown. *E. gyrosa* was proposed as the type species of the genus *Endothia* based on *Sphaeria gyrosa* Schw. from Georgia, North American in 1849 (Fries 1849). *E. gyrosa* is common on many deciduous tree species, including *Quercus*, *Acer*, *Fagus* and *Liquidambar* in southeastern North America (Barr 1978), but only on eucalypts in Australia (Walker *et al.* 1985; Old *et al.* 1986; Sankaran, Sutton and Minter 1995). It has not been found on *Quercus* or *Liquidambar* which are present as ornamentals in Australia. As pointed out by Walker (1996), *E. gyrosa* may be a recent introduction from North America because of its narrow host range in Australia. It could also be a native fungal species which has been overlooked on other plant hosts apart from eucalypts. Molecular comparisons of *E. gyrosa* isolates from different Australian origins and different countries may help in answering the problem.

Many current DNA analyses are based on the polymerase chain reaction (PCR). With PCR primers amplify a minute quantity of DNA. The amplified product(s) can be detected directly on an agarose gel or undergo further analytical techniques (eg. restriction endonuclease digestion or sequencing).

Restriction analysis of the PCR amplified ITS region of rDNA (using the universal primers ITS1 ITS2, ITS3, ITS4 *etc.*) has been successfully used to determine genetic variation among fungal species. These ITS repeat units evolve rapidly and vary among species within a genus (White *et al.* 1990).

RAPD (random amplified polymorphic DNA) analysis provides a more rapid means of comparing overall genetic similarity among different individuals generally for studies of population structure and taxonomy. It is a technique which amplifies DNA using a single arbitrarily designed primer (Williams *et al.* 1990). Complex patterns of the amplification results are used for comparisons. The genetic variability in many plant pathogenic fungi has been investigated with RAPD analysis (Weising *et al.* 1995).

Cultural morphology and vegetative compatibility

Old and Dudzinski (1991, unpublished data) noticed two types of colony colour among *E. gyrosa* isolates, eg. orange-coloured and grey-colored isolates. In a preliminary investigation of naturally infected trees in East Gippsland, Victoria, isolates of *E. gyrosa* with different colony colour were found to be vegetatively incompatible even from the same tree (Kubono 1991, unpublished data). Apart from Kubono, there have been no studies of the cultural variation and vegetative compatibility with *E. gyrosa*. Such studies with a closely related fungus *Cryphonectria parasitica* and other ascomycetes, *Aspergillus*, *Fusarium* and *Neurospora* as well as the basidiomycete *Armillaria*, have been numerous, especially in the area of VC groups (Shaw and Kile 1991; Leslie 1993; Carlile and Watkinson 1994; Causin *et al.* 1995).

Contact between hyphae of different strains of the same species may result in vegetative incompatibility (VIC) or heterokaryon incompatibility (HET). Since vegetative incompatibility is based on genetic differences it is a form of heterogenic incompatibility which can also occur in the sexual phase, although sexual heterogenic incompatibility has received less study than vegetative heterogenic incompatibility (Carlile and Watkinson 1994).

Vegetative compatibility is controlled by both allelic and nonallelic systems (Glass and Kuldau 1992). Allelic determination of VC prevents a compatible interaction between two strains that have different alleles at a particular HET locus. Only individuals with identical genotypes at all HET loci are compatible. Nonallelic vegetative incompatibility occurs when alleles of genes at two separate loci interact incompatibly as in *Podospora anserina* (Ces. ex Rob.) Niessl. Compatible interactions result in the merging of the two mycelia growing together on agar media. Hyphal fusions occur and nuclei may exchange between the two mycelia. Incompatible interactions often result in barrage reactions (*sensu* Leslie 1993) with clear zones produced by lysis of the hyphae between the two opposed mycelia. Stimulation of asexual sporulation may occur along the borders of the barrage zone.

The presence of vegetative incompatibility systems in filamentous ascomycetes has several practical consequences for plant pathologists. VC groups have been used to study population dynamics of plant pathogenic fungi as well as nonpathogenic isolates. VC group identification has been useful in determining the source of races new to a particular geographical area and the presence of large numbers of VC groups is considered an indicator of genetic diversity within a population (Glass and Kuldau 1992). In *C. parasitica*, for example, the large number of VC groups found within the same stand in the USA limits the effectiveness and ease of biological control with hypovirulent strains which must be of the same VC group for successful transmission of the hypovirulence. In Europe far fewer VC groups are present and biological control is more successful.

Fungicide response

Response to fungicide is exploited to detect and assess variation in populations of fungi (Caten 1996). Resistance to fungicide, especially systemic fungicides, such as benzimidazoles is known to arise through a mutation. Fungal populations can be classified into groups on basis of their sensitivity to a particular fungicide. For example, 66 isolates of *Verticillium fungicola* (Preuss) Hassebr, a fungal pathogen, assessed for their sensitivity to benomyl, thiabendazole and chlorothalonil, were categorised as sensitive, slightly resistant, moderately resistant or highly resistant (Bonnen and Hopkins 1997).

Infection ability of conidia and ascospores

The teleomorph of *E. gyrosa* has not been reported in Western Australia (Shivas 1989, Shedley pers comm) whereas both the anamorph and teleomorph are very common in south-east Australia and Tasmania (Old *et al* 1986; Yuan 1989; Wardlaw 1998; Chapter 1). In the 1993 severe *E. gyrosa* epidemic, Wardlaw (1998) only observed the teleomorph. In subsequent years, superficial infections observed on rough barked trees were only sporulating sexually. It might be assumed from the above observations that both asexual and sexual spores are effective in dispersal and infection.

The balance and interplay between asexual reproduction has been discussed by Chamberlain and Ingram (1997). It is clear that a trade-off operates between asexual and sexual reproduction in fungi. The balance of resources allocated to the competing demands of the two modes of reproduction can be determined genetically and can also be regulated by numerous environmental factors. Sexual structures and sometimes the sexual spores themselves are more durable, allowing the organism to resist unfavourable conditions. In some fungal groups the sexual structures also allow for better dispersal.

One of the first step to understand the role of asexual and sexual reproduction in the life cycle and epidemiology of *Endothia gyrosa* is to develop and test a technique to artificially inoculate both ascospores and conidia under controlled conditions, comparing lesion development from both spore types and mycelial inoculations. Successful inoculation with the conidia of *E. gyrosa* had been obtained by superficial bark wounds of 16 year old trees (Chapter 2, Section 2.3.2.4). Research in Chapter 2 (Section 2.3.1) also showed that tests with seedlings gave rise to results which were comparable to those with older trees as regards lesion development by different *E. gyrosa* isolates. Since better experimental control can be achieved by the use of seedlings in the greenhouse an artificial inoculation with conidia, ascospores and mycelium was conducted on basis of the artificial inoculation of seedlings.

Intraspecific variation in pathogenicity

Old *et al.* (1986, 1990) have shown the existence of pathogenic variability between isolates of *E. gyrosa* from southeast Australia on a range of eucalypt species. They found some isolates were more pathogenic than others to the eucalypts tested. In the pathogenicity tests presented in Chapter 2 significant pathogenic variation as measured by lesion size was also observed among the three *E. gyrosa* isolates, TAS1, TAS3 and TAS9 from a single location of Tasmania. TAS1 was the most pathogenic isolate, with TAS9 intermediate and TAS3 the least pathogenic.

Wardlaw (1998) attributed the disease epidemic in the *E. nitens* plantation in Tasmania to either a more virulent isolate of *E. gyrosa* or an inherently more

susceptible host or a particular isolate of the pathogen interacting with a particular host provenance. The pathogenicity tests with the above-mentioned isolates of *E. gyrosa* and other stem canker fungi (Chapter 2) have shown that rough bark predisposes infection via cracks. However this does not preclude the presence at the epidemic site of more virulent strains of *E. gyrosa*. The pathogenicity of isolates from this epidemic site and from elsewhere must be compared.

3.2. METHODS AND MATERIALS

3.2.1 INTRASPECIFIC VARIATION IN PATHOGENICITY

3.2.1.1 Fungal isolates

Sixteen Australian isolates of *E. gyrosa* were used. Eight originated from Tasmania, with 7 isolated from *E. nitens*. Six isolates originated from southeast Australia and two from Western Australia (Table 3.2-1).

Table 3.2-1: Origin of *Endothia gyrosa* or *Endothiella gyrosa* isolates tested for pathogenicity

Isolate No.	Herbarium No.[A]	Host	Locality	Isolation Date
ACT1	E10	*E. pauciflora*	Corin Rd, ACT	23/05/82
ACT2	E14	*E. viminalis*	Brindabella, ACT	-
NSW1	E8	*E. delegatensis*	Batlow, NSW	03/03/82
NSW2	E11	*E. saligna*	Currowan SF, NSW	-
VIC1	Orbost71	*E sieberi*	Orbost, VIC	23/08/89
VIC2	E9	*E. viminalis*	Wombat SF, VIC	14/05/82
WA1	WA20	*E. maculata*	?, WA	16/08/84
WA2	WA60	*E. wandoo*	?, WA	20/05/85
TAS1	ECF11	*E. nitens*	Ridgley, Burnie, TAS	25/05/95
TAS3	ECF1	*E. nitens*	Ridgley, Burnie, TAS	25/05/95
TAS4	ECF8	*E. nitens*	Ridgley, Burnie, TAS	25/05/95
TAS7	ECF4	*E. nitens*	Ridgley, Burnie, TAS	25/05/95
TAS8	ECF13	*E. nitens*	Ridgley, Burnie, TAS	13/05/95
TAS9	ECF25	*E nitens*	Ridgley, Burnie, TAS	25/05/95
TAS10	ECF27	*E. nitens*	Ridgley, Burnie, TAS	25/05/95
TAS11	E18	*E. regnans*	Lone Star, TAS	?/?/82

[A]All ECF isolates are held by the authors at CSIRO, Forestry & Forest Products, Tasmania (TAS), and the isolates with E, Orbost and WA designation at CSIRO, Forestry & Forest Products, Canberra, Australian Capital Territory (ACT). NSW = New South Wales; VIC = Victoria; WA = Western Australia

The isolates TAS1, TAS3 and TAS9 were used in Chapter 2 and they were included in this study for further comparison.

All the Tasmanian isolates were isolated from canker tissue in different trees at the same or different localities.

3.2.1.2 Mycelial Inoculum

Preparation of mycelial inoculum was the same in the general pathogenicity tests in Chapter 2 (Section 2.2.2.1).

3.2.1.3 Seedlings

The same *E. nitens* seedlings of provenance NE-1 as those in Chapter 2 were used in this test (see Chapter 2, section 2.2.3).

For *E. globulus*, the origin and age of the seedlings were the same as described in Chapter 2 (Section 2.2.3).

All seedlings were maintained in the shade house as described in Chapter 2.

3.2.1.4 Inoculation with mycelium-bran

The same cambium-deep inoculation technique was applied as in Chapter 2 (Section 2.2.5).

3.2.1.5 Experimental design

Twelve-month-old seedlings of *E. nitens* and *E. globulus* were tested. Seedlings of each species were inoculated with 16 isolates *E. gyrosa* (Table 3.2-1). A total of 85 seedlings [(16 isolates + 1 control) x 5 seedlings] were used for each species.

Inoculations for *E. nitens* were carried out in January, 1996. The first measurements of canker lesions were made two months after inoculation and then these seedlings were maintained in the shade house and observed until seven months after inoculation.

The inoculations of *E. globulus* were in June 1997 (winter) and the measurements of canker lesions were made seven months after inoculation.

3.2.1.6 Canker evaluation, reisolation of fungus and analysis of data

Methods for the evaluation of cankers, fungal reisolation and data analysis were the same as those for the general pathogenicity tests in Chapter 2 (Sections 2.2.7, 2.2.8 & 2.2.10).

3.2.2 INFECTION ABILITY OF CONIDIA AND ASCOSPORES

3.2.2.1 Isolates

The teleomorph for each of the isolates (Table 3.2-2) was collected from cankers in the field. TAS12 and TAS13 were collected from a *E. nitens* plantation with severe stem cankers associated with *E. gyrosa*. TAS14 was collected from *E. regnans* F. Muell plantation with a large (ca.30 cm long) canker at Westfield, southern-central Tasmania.

All three isolates were single ascospore isolates. Ascospores were suspended on sterile slides by mixing them with sterilised water and then spread on a 2% thin layer of water agar (WA) in Petri dishes. After 1-2 days of incubation on WA, mycelium emanating from single spores was transferred to potato dextrose agar using a flat-ended transfer needle. Pure single-spore sub-cultures of these isolates were transferred onto PDA slopes in plastic vials and stored at 4°C.

Table 3.2-2: Origin of isolates of *Endothia gyrosa* or *Endothiella gyrosa* associated with *Eucalyptus* spp. used for a comparison of the infection ability of conidia and ascospores

Isol. No.	Herb. No.[A]	Host	Locality	Iso. Date
TAS12	ECF232	*E. nitens*	Ridgley, Burnie, TAS	14/10/96
TAS13	ECF233	*E. nitens*	Ridgley, Burnie, TAS	14/10/96
TAS14	ECF234	*E. regnans*	Westfield, TAS	14/10/96

[A]All ECF isolates are held at CSIRO, Forestry & Forest Products, Tasmania (TAS)

3.2.2.2 Seedlings

Seventeen-month-old seedlings of NE-13, NE-14, NE-15 were used for spore suspension inoculations (Table 3.2-3). The seedlings were bought from Woodlea Nursery, Tasmania in 15 cm plastic pots and maintained in the shade house as described in Chapter 2 (Section 2.2.3).

Table 3.2-3: Seedlings of *Eucalyptus nitens* provenances used for ascopore and condial inoculations

No.	Provenance	Locality
NE-13	Toorongo	Upper Thompson, Victoria
NE-14	Toorongo	Toorongo Plateau, Victoria
NE-15	Toorongo	Mt Erica, Victoria

3.2.2.3 Inoculum

(a) Ascospore inoculum

Freshly collected cankered bark with *E. gyrosa* perithecia was cut into small pieces (ca. 2 mm^3) and soaked in 50 ml of sterile water for 20 minutes. After being macerated in a blender ("Ronson", model 8344) for 20-30 seconds, the mixture was filtered through gauze to remove the larger pieces of bark, and then 2-3 times through tissue paper. The filtered solution was centrifuged at a speed of 1000-2000 rpm to collect spores. The spores were diluted with sterile water to either 2 x 10^7 spores/ml or 0.5 x 10^7 spores/ml. The spore concentration was estimated as in Chapter 2 (Section 2.2.2).

(b) Conidial inoculum

Conidial inoculum was prepared as in Chapter 2 with two concentrations, 2 x 10^8 spores/ml and 0.5 x 10^8 spores/ml. The spore concentration was estimated as in Chapter 2 (Section 2.2.2).

(c) Mycelial inoculum

Mycelial inoculum was prepared as in Chapter 2 (Section 2.2.2).

3.2.2.4 Inoculation with conidial and ascospore suspension

Seedling stems for spore inoculation were wounded to the depth of the cambium using a cork-borer (3mm in diameter) which was surface-sterilized with 95% ethanol before and between use. A drop of spore suspension (1µl) was applied to a wound using an automatic pipettor. The two concentrations prepared resulted in either 5,000 or 20,000 ascospores or either 50,000 or 200,000 conidia being inoculated in each wound. Three wounds were made along stems of each seedling at a height of 10-20 cm above soil level, leaving 10-20 cm between each wound. The upper two wounds were inoculated with the two different concentrations of spore-suspension and the lowest one served as a control. Sterile water (1µl) was applied to a wound as a control. As soon as they were applied the drops of spore-suspension were completely absorbed into the plant tissue. The inoculated wounds were wrapped with plastic film.

3.2.2.5 Experimental design

Seventeen-month-old seedlings of three *Eucalyptus nitens* races (NE-13, NE-14, NE-15) were used for the test in the shade house at Hobart.

Ascospore and conidial suspensions of three *Endothia gyrosa* isolates, TAS12, TAS13 and TAS14 were inoculated on stems of the potted seedlings. For comparison, seedlings were also inoculated with mycelium-mixed wheat/rice bran using the inoculation technique described in Chapter 2 (Section 2.2.5).

Each treatment (eg. isolate x provenance x inoculum type) was replicated five times and was distributed in five separate blocks. Within each block, the seedlings were randomly arranged as above. A total of 135 seedlings (3 races x 3 isolates x 3 inoculum types x 5 replicates) were inoculated.

The experiment was carried out in spring (Nov. 1996). The wounds were observed over a period of 4 months and the final lesion measurements were carried out 4 months after inoculation

3.2.2.6 Canker evaluation, reisolation of fungus and analysis of data

Evaluation of canker, reisolation of fungus and analysis of data were the same as those for the general pathogenicity tests in Chapter 2 (Section 2.2.7, 2.2.8 & 2.2.10).

3.2.3 COLONY MORPHOLOGY, FUNGICIDE RESPONSE AND VEGETATIVE COMPATIBILITY GROUPS

3.2.3.1 Isolates

A total of 133 isolates of *E. gyrosa*, 95 from Tasmania (TAS), 6 from Australian Capital Territory (ACT), 6 from New South Wales (NSW), 7 from Victoria (VIC), 7 from Western Australia (WA), 2 from Italy, 3 from USA and 7 from South Africa were available (Appendix 5). All but one isolate from Tasmania (ECF234) were obtained by isolation from visible conidial masses on stems or from cankered tissue. ECF234 was a single ascospore isolate.

Table 3.2-4: Origin of sixteen *Endothia gyrosa* isolates

No.	Isolate	Herbarium No.[A]	Collection Site	Host
1	TAS1	ECF11	Tewkesbury, Tas.	*Eucalyptus nitens*
2	TAS2	ECF9	Tewkesbury, Tas.	*E. nitens*
3	TAS3	ECF1	Tewkesbury, Tas.	*E. nitens*
4	TAS4	ECF8	Tewkesbury, Tas.	*E. nitens*
5	TAS5	ECF197	Camden, Tas.	*E. nitens*
6	TAS6	ECF202	Camden, Tas.	*E. nitens*
7	ACT1	E10 (DAR 44532)	Corin Rd, ACT	*Eucalyptus pauciflora*
8	NSW1	E8 (DAR 51155)	Batlow, NSW	*Eucalyptus delegatensis*
9	VIC1	Orbost 71	Orbost, Vic.	*Eucalyptus sieberi*
10	WA1	WA20	?, WA	*Eucalyptus maculata*
11	WA2	WA60	?, WA	*Eucalyptus wandoo*
12	USA1	ATCC48192	Virginia, USA	*Quercus palustris*
13	USA2	CBS.509.76	Virginia, USA	*Quercus palustris*
14	ITA1	CBS.251.54	La Coruna, Italy	*Castanea crenata* var. *tamba*
15	SA1	CRY 62	Sabie, South Africa	*Eucalyptus grandis*
16	SA2	CRY 94	Sabie, South Africa	*Eucalyptus grandis*

[A]All ECF isolates are held by the authors at CSIRO, Forestry & Forest Products, Tasmania;
The isolates with E, Orbost, WA designation are held at CSIRO, Forestry & Forest Products, Canberra, ACT.
ATCC = American Type Culture Collection at Maryland, USA;
CBS = Centraalbureau voor Schimmelculturees at Baarn, Netherlands;
DAR = Plant Pathology Herbarium at Orange NSW, Australia.
CRY isolates are provided by Prof. M. Wingfield, Dept. of Microbiology & Biochemistry Univ. of the Orange Free State, South Africa.

All 133 isolates were examined for determination of differences in colony morphology. For studies of response to fungicide and vegetative compatibility (VC), sixteen of these 133 isolates, 2 from America, 11 from Australia (6 from Tasmania and 5 from mainland Australia), 1 from Italy and 2 from South Africa were used (Table 3.2-4).

3.2.3.2 Colony morphology

Colony morphology was determined on malt extract agar (MEA) (Saunders' Malt Extract, Australia). Isolates were recovered by transfer of stock cultures onto MEA. Petri dishes (9-cm diam) containing 3% MEA were inoculated with 5-day-old cultures and incubated at 22°C in the dark.

Cultures of the selected 16 isolates in Table 3.2-4 were also exposed to light and subcultured several times under the same incubation conditions as above to see if there were any changes of colony types.

Colonies were scored once a week for four weeks starting at 10 days after inoculation. Characters of colonies scored were as follows:

1. colour: white, grey, buff, vitelline, orange or reddish brown;
2. density: fluffy or appressed, thin or dense.

Data were analysed using the numerical taxonomy package NTSYS-pc version 1.8 (Rohlf 1993). A similarity matrix was produced with the SIMQUAL program using 'the simple matching coefficient' (Sokal and Sneath 1963). Cluster analysis was performed through the SAHN cluster program using the unweighted pair-group arithmetic average (UPGMA) and a dendrogram was constructed using the sub-program "Tree".

3.2.3.3 Response to fungicides

A slightly modified method of Bonnen and Hopkins (1997) using radial growth was used to assess sensitivity of the *E. gyrosa* isolates to fungicides. Disks of agar bearing cultures 2-mm^2 in area that had been growing for two weeks on PDA plates were transferred onto PDA plates amended with fungicides. Three dishes of each fungicide were inoculated with each of sixteen isolates. Three extra dishes containing PDA were inoculated with each isolate as controls. The plates were incubated in darkness at 22°C. Radial growth was measured at 1 week intervals for 21 days. Growth measurements were given as a percentage of control growth. The measurements during the first two weeks were selected for data analysis.

The isolates were grouped based on their response (radial growth) to each fungicide as follows: sensitive (S) = 0-20% of control growth, slightly resistant (SR) = 20-40% of control growth, moderately resistant (MR) = 40-60% of control growth and highly resistant (HR) = 60-80% of control growth.

The fungicides were benomyl (Benlate, 50% active constituent, [a.c.], Du Pont (Australia) Ltd.), chlorothalonil (Brave 500, 50% a.c., Agchem Inatec Ltd.) and thiabendazole (Sigma, 99% a.c.). All fungicides were used at a concentration of 50 mg/L in potato extrose agar (PDA) (Difco). No attempt was made to use other concentrations.

3.2.3.4 Vegetative compatibility groups

Two different media, 2% MEA and PDAvc media were used for determination of VC groups.

VC-groups were determined on 2% MEA. Test pairs of 16 isolates in all possible combinations were placed ca. 8 mm apart, 24 pairs per dish (9-cm diam) as described by Anagnostakis (1977). They were incubated at 21°C in darkness and examined weekly for 4 weeks.

To confirm VC reactions, a method developed by Powell (1995) for *Cryphonectria parasitica* was used. All the sixteen isolates were paired at 21°C in darkness on PDAvc medium (Difco potato extrose agar supplemented with 0.2g/L tannic acid, 0.1g/L methionine, 2 mg/L biotin, 2 mg/L thiamine and 5g/L extra agar) containing 50 mg/L bromocresol green (pH 3.8 yellow; pH 5.4 blue-green). Plugs 2-mm^2 were removed from the margin of actively growing (one week old) colonies and placed on 9-cm plastic petri dishes. Three plugs each from a different isolate were spaced 1 cm from each other near the center of plates. Two plugs of the same isolate were placed in separate plates for self-pairings. The zones of confrontation were screened for colour changes ten days after inoculation.

To check hyphal anastomoses within VC-groups under microscope, autoclaved cellophane membranes were placed between the converging colony fronts of the paired isolates as described by Newhouse and MacDonald (1991).

Agar blocks (3 mm in diam.) containing hyphal tips of different isolates to be paired were taken from 5-day-old cultures on PDA. Two blocks were aseptically placed 1 cm apart, with the hyphal tips oriented toward one another. Three pairings were made per plate. The paired cultures were incubated at 21°C in darkness. Twelve hours later, 5 x 7 mm cellophanes autoclaved in potato extrose broth were placed between the converging colony fronts of the paired isolates. The cultures were incubated at the same conditions as above for 1-2 days till anastomoses were observed under microscope.

3.2.4 DNA POLYMORPHISM

3.2.4.1 Fungal isolates

Sixteen isolates listed in Table 3.2-4 were used in the present study.

3.2.4.2 DNA extraction

Cultures were grown on liquid Emerson's yeast extract (15g potato starch, 4 g yeast extract, 1 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$) and liquid malt extract (Saunders' Malt Extract, Australia) for 7-10 days.

The DNA extraction was based on the protocol used by Dr. R. Vilgalys, Duke University, USA (personal comm.,1996).

- Mycelium was collected by filtration (filter paper) and ground to a fine power in liquid nitrogen using a mortar and pestle.
- Small amounts of the powdered mycelium was dispensed into 1.5 ml eppendorf microcentrifuge tubes (up to the top of the conical portion of the tube).
- Mycelium was suspended in 500 µl of extraction buffer [1% (w/v) CTAB (cetyl-trimethyl-ammonium bromide) (Sigma H5882), 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (ethylenediamine tetraacetic acid), 700 mM NaCl] containing 1% (v/v) 2-mercaptoethanol.
- After the tubes were incubated in a water bath at 65 °C for 30-60 min, the samples were extracted twice, first with 500µl of 25:24:1 phenol:chloroform:isoamyl alcohol (v:v:v) and second with 24:1 chloroform:isoamyly alcohol (v:v).
- Each extraction was followed by centrifugation (12 min, 13000 rpm), and the aqueous phase transferred to new tubes.
- To digest the RNA (ribonucleic acid), 25 µl of 10 mg/ml RNAse (ribonuclease) stock solution in 50 mM Tris-HCl (pH 7.5), 200 mM NCI, 300mM KCl and 50% Glycerol (DNAse free) was added to each tube and incubated at 37°C for 30 min.
- The DNA was precipitated with 0.6 volumes of cold isopropanol at -20 °C overnight and pelleted by centrifugation at 13000 rpm for 5 min.

- The DNA pellets were washed briefly (2 min) with 100 µl of cold 70% ethanol, dried 30-40 min in a vacuum oven at 35 °C and resuspended in 50 µl of TE buffer (10 mM Tris HCI and 1 mM EDTA, pH 8.0).
- The DNA samples were quantified with a TKO-100 Hoefer fluorimeter (Hoefer Scientific Instruments, San Francisco, CA) and adjusted to 30-50 ng/µl in TE buffer as stock solutions.

3.2.4.3 PCR (polymerase chain reaction) amplification of the ITS region

(a) Primers

The rDNA (ribosomal DNA) ITS (internal transcribed spacer) region containing ITS1 and ITS2 and the intervening 5.8S rDNA gene was amplified using the universal primer pairs, ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) described by White *et al.* (1990).

(b) Reaction template

The reaction for the PCR was carried out in 25 µl volumes containing 2.5 µl of 10x reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.5), 1.0% Triton®x-100], 1.5 µl of $MgCl_2$ (25mM), 3 µl of dNTPs (deoxynucleotide triphosphates) (2 mM), 2.5 µl of BSA (bovine serum albumin) (1µg/µl), 1 µl of each primer (10 µM or 10 pmol), 0.2 µl of *Taq* Polymerase, 12.3 µl sterile, deionised distilled water (ddH_2O), and 1µl of DNA (10ng/µl). A layer of light mineral oil (Sigma) was applied to prevent evaporation. The negative control contained 1 µl of sterile ddH_2O instead of DNA.

(c) DNA amplification

A thermal cycler (PTC-100 Programmable Thermal Controller or/and FTS-960 Thermal Sequencer) was used for the DNA amplification using the following program: step (1) initial denaturing for 5 min at 94 °C; step (2) denaturing for 30 sec at 94 °C; step (3) annealing for 1 min at 50 °C; step (4) extension for 2 min at 72 °C; step (5) final extension for 8 min at 72 °C. The steps 2-4 were repeated 35 times.

The amplification program was based on the protocol used by Moricca and Ragazzi (1998).

(d) Restriction Enzyme Digestion

DNA obtained by amplification of the ITS region was analysed by restriction enzyme digests. Digests of 20 µl containing 10 µl of amplified PCR product, 0.5 µl of each enzyme and 2 µl of its 10x buffer, and 7.5 µl of water (ddH_2O) were incubated at 37 °C for 2 hrs to over night.

The following enzymes which cut DNA at specific 4-6 base sites were used: *Alu* I, *Cla* I, *Dpn* II (Biolabs), *Hae* III, *Hin* fI (Biolabs) and *Msp* I (Pharmacia).

3.2.4.4 PCR amplification of nuclear DNA for RAPD analysis

(a) Primers

A total of 23 different single 10-oligomer random primers purchased from the University of British Colombia (UBC) and Operon Technologies, Inc. (OP) were screened for polymorphism among samples. Thirteen primers which showed polymorphism in samples were chosen:

UBC-04 (CCT GGG CTG G)	UBC-05 (CCT GGG TTC C)
UBC-23 (CCC GCC TTC C)	UBC-30 (CCG GCC TTA G)
UBC-81 (GAG CAC GGG G)	UBC-90 (GGG GGT TAG G)
UBC-215 (TCA CAC GTG C)	UBC-218 (CTC AGC CCA G)
UBC-230 (CGT CGC CCA T)	UBC-234 (TCC ACG GAC G)
UBC-237 (CGA CCA GAG C)	UBC-243 (GGG TGA ACC G)
OP-B08 (GTC CAC ACG G).	

(b) Reaction template

RAPD reactions were carried out in 20 µl volumes containing 2 µl of 10x reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.5), 1.0% Triton®x-100], 2 µl of 2 mM dNTPs, 2.4 µl of 25mM $MgCl_2$, 3 µl of 1µg/µl BSA, 0.2 µl of 50 pmol of primer, 6.1 µl of sterile, deionised distilled water (ddH_2O), 0.3 µl of *Taq* Polymerase and 4.0 µl of 5 ng/µl DNA. The control contained 4.0 µl of sterile ddH_2O instead of DNA. Thirty µl sterile mineral oil was overlaid on each reaction.

(c) DNA amplification

A thermal cycler (PTC-100 Programmable Thermal Controller) was used for the DNA amplification using the following program: step (1) initial denaturing 2 min at 94 °C; step (2) denaturing 1 min at 94 °C; step (3) annealing 1 min at 35 °C; step (4) extension 2 min at 72 °C; step (5) final extension 5 min at 72 °C. The steps 2-4 were repeated 39 times.

3.2.4.5 Electrophoresis

Following amplification and digestion, the PCR and digest products were separated by electrophoresis using 1.5% agarose gel in TBE running buffer (0.09 M Tris-borate, 0.09 boric acid and 0.002 M EDTA, pH 8.0) at 70 V.

Six µl of each reaction product and 4 µl of a 100bp ladder (Promega Madison and Bresatec DMW-100L) was mixed with 3.0 µl of 5x GLB loading buffer (Promega) (0.25% bromophenol blue, 25% sucrose, 6.25 mM EDTA, 1.25% SDS). Gels were stained with ethidium bromide (0.5 µg/ml gel), examined under ultraviolet light and photographed using a Polaroid camera and Polaroid 665 film.

3.2.4.6 Analysis of RAPD Data

RAPD bands were scored as present (1) or absent (0). Data were analysed using the numerical taxonomy package NTSYS-pc version 1.8 (Rohlf 1993). A similarity matrix was produced with the SIMQUAL program using 'the simple matching coefficient' (Sokal and Sneath 1963). Cluster analysis was performed through the SAHN cluster program using the unweighted pair-group arithmetic average (UPGMA) and a dendrogram was constructed using the sub-program "Tree".

3.3 RESULTS

3.3.1 INTRASPECIFIC VARIATION IN PATHOGENICITY

3.3.1.1 Pathogenic variability to *E. nitens* seedlings

(a) Observations at two months after inoculation

Variation in pathogenicity (as expressed by external lesion length and tangential spread) was observed among the 16 isolates tested on 12-month-old seedlings of *E. nitens* (NE-1) (Fig. 3.3.1-1 & 3.3.1-2).

Lesions produced by fourteen isolates (ACT2, NSW2, VIC1, VIC2, WA1, WA2, TAS1, TAS3, TAS4 and TAS7 through TAS11) were significantly ($P \leq 0.05$) longer than those of controls (Fig. 3.3.1-1). Isolate ACT1 did not produce any lesions significantly larger than the controls. Similarly lesions on stems inoculated with NSW1 were not significantly longer than those of controls, but lesion tangential spread was significantly greater than the control (Fig. 3.3.1-2).

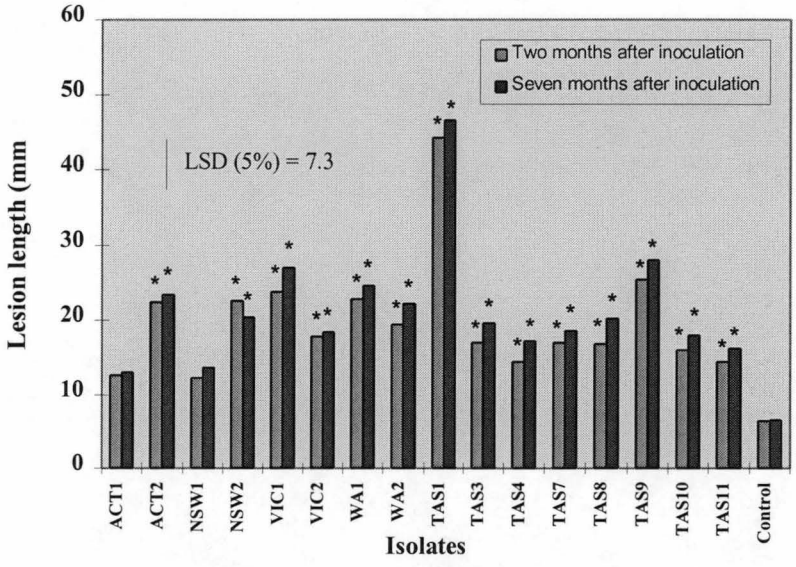


Fig. 3.3.1-1: Mean length (mm) of external canker lesions on stems of *Eucalyptus nitens* (NE-1) seedlings inoculated with 16 isolates of *Endothia gyrosa*, assessed 2 and 7 months after inoculation

Bars with an asterisk are significantly different to the control.

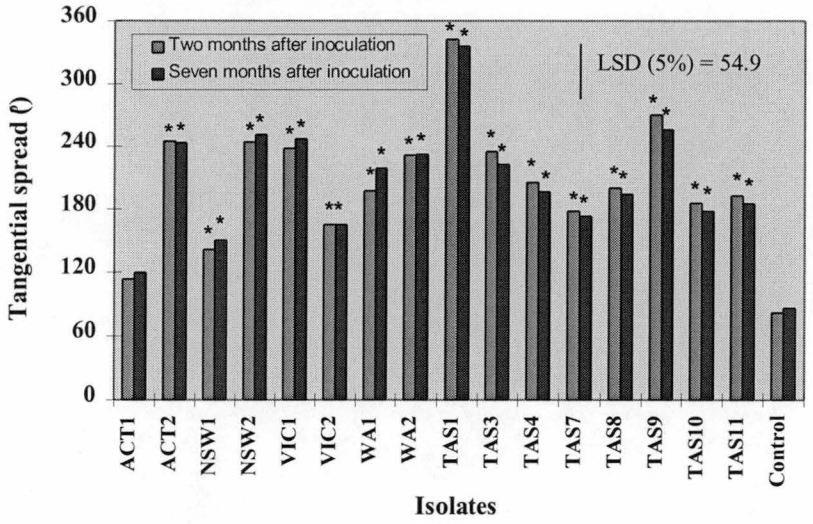


Fig. 3.3.1-2: Mean tangential spread of lesions on stems of *Eucalyptus nitens* (NE-1) seedlings inoculated with 16 isolates of *Endothia gyrosa*, assessed 2 and 7 months after inoculation

Bars with an asterisk are significantly different to control.

Among the isolates tested, TAS1 produced longest lesion lengths and tangential spreads which were significantly different to those produced by other isolates. The lesions had half girdled stems 2 months after inoculation. TAS9 produced the second longest lesions and tangential spreads, but the lesions were not significantly different to those produced by VIC1, WA1, NSW2, ACT2 and WA2.

Callus development was observed within 3 weeks after inoculation in both the inoculated wounds and controls. All of the wounds in the controls were found completely occluded at the first assessment (Table 3.3.1-1). Lesions on all of the seedlings inoculated with ACT1 were also completely occluded 2 months after inoculation and there were no fruiting bodies present on the surface of the lesions (Table 3.3.1-1). Four out of five lesions produced by VIC2, two by NSW1 and one by TAS10 had occluded 2 months after inoculation. All of the lesions produced by ACT2, NSW2, VIC1, WA1, WA2, TAS1, TAS3, TAS4, TAS7, TAS8, TAS9 and TAS11 remained open.

Orange coloured fruiting bodies were found abundantly on surface of the lesions produced by ACT2, NSW2, VIC1, WA1, TAS1, TAS3, and TAS9 (Table 3.3.1-1).

Table 3.3.1-1: *Eucalyptus nitens* (NE-1) seedlings inoculated with 16 isolates of *Endothia gyrosa*: number of lesions occluded and with fruiting bodies present on the surface of lesions

	No. of lesions occluded		No. of lesions with fungal fruiting bodies	
Isolates	2 months	7 months	2 months	7 months
ACT1	5[A]	5	0[A]	0
ACT2	0	4	3	5
NSW1	2	5	0	0
NSW2	0	1	2	4
VIC1	0	1	5	5
VIC2	4	5	0	0
WA1	0	1	5	5
WA2	0	3	0	2
TAS1	0	1[B]	5	5
TAS3	0	4	5	5
TAS4	0	5	0	1
TAS7	0	5	0	0
TAS8	0	5	0	0
TAS9	0	2	5	5
TAS10	1	5	0	3
TAS11	0	5	0	4
Control	5	5	0	0

[A]Number of lesions occluded or lesions with fruiting bodies/total 5 lesions (seedlings) inoculated with each isolate.
[B]One seedling was dead due to inoculation of TAS1.

(b) Observations at 7 months after inoculation

Between isolate differences in lesion extent observed at 7 months were similar to those assessed 2 months after inoculation. Analysis of variance showed no significant ($P \leq 0.05$) differences between lesion length and tangential spread at 2 months and 7 months after inoculation (Appendix 6), although all the lesions had increased slightly in length and tangential spread with time (Fig. 3.3.1-1 & Fig. 3.3.1-2).

The lesions produced by TAS1 were still significantly larger than those produced by other isolates seven months after inoculation. Lesions nearly girdled the stems and sapwood was exposed. One seedling inoculated with this isolate was dead.

Most of the larger lesions produced by NSW2, VIC1, WA1 and TAS1 had not occluded at 7 months (Table 3.3.1-1). Smaller lesions produced by NSW1, VIC2, TAS4, TAS7, TAS8, TAS10 and TAS11, however, were fully occluded at this later date.

No fruiting bodies were found on the surface of smaller lesions produced by ACT1, NSW1, VIC2, TAS7 and TAS8 (Table 3.3.1-1).

No necrotic discolouration of internal tissue of control wounds was observed beyond inoculation points.

Internal discolouration was strongly correlated (r =0.87, P<0.001) with external lesion length 7 months after inoculation (data not presented). For example, the mean internal discolouration of sapwood associated with lesions by TAS1 was 69 mm (Fig. 3.3.1-8*a*).

Table 3.3.1-2: Percentage of reisolation from seedling stems of *Eucalyptus nitens* (NE-1) and *E. globulus* inoculated with 16 isolates of *Endothia gyrosa* assessed seven months after inoculation

Isolates	*E. nitens*	*E globulus*
NSW2	100 a	100 a
VIC1	100 a	76 c
WA1	100 a	100 a
WA2	100 a	100 a
TAS1	100 a	100 a
TAS3	100 a	100 a
TAS4	100 a	100 a
TAS8	100 a	88 b
TAS9	100 a	100 a
TAS10	100 a	88 b
TAS7	96 b	100 a
TAS11	96 b	100 a
ACT2	96 b	88 b
VIC2	92 c	60 de
NSW1	76 d	56 e
ACT1	56 e	64 d
Control	0 f	0 f
LSD (5%)	3.5	7.3

* % Re-isolation = number of wood fragments yielding fungi inoculated/total numbers of fragments cut from lesions induced by each of the fungal isolates inoculated. Means sharing the same letter(s) are not significantly different.

All the isolates were successfully isolated from 56-100% of wood fragments excised adjacent to inoculation points (Table 3.3.1-2).

3.3.1.2 Pathogenic variability of *E. gyrosa* to inoculated *E. globulus* seedlings

Measurements of lesions on *E. globulus* seedlings were made 7 months after inoculation.

Lesions resulting from the inoculation of *E. globulus* seedlings closely resembled those on *E. nitens* (Fig. 3.3.1-3 & 3.3.1-4). The lesions on *E. globulus* seedlings appeared to spread longitudinally further than those on *E. nitens* seedlings during the same period of development. Significant ($P \leq 0.01$) variation in lesion length and tangential spread was detected among the sixteen isolates from the same or different geographic origins. Some differences in the ranking of isolates with regard to their pathogenicity occurred, and all isolates induced lesions significantly different from controls.

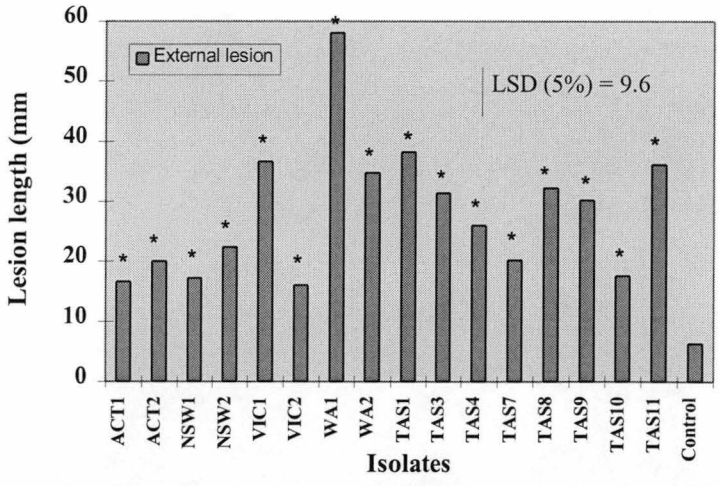


Fig. 3.3.1-3: Mean length (mm) of external canker lesions on stems of *Eucalyptus globulus* seedlings inoculated with 16 isolates of *Endothia gyrosa*, assessed seven months after inoculation

Bars with an asterisk are significantly different to control.
The mean length for TAS1 was a mean of 4 replicates, since one seedling was dead due to the inoculation.

All sixteen isolates produced significantly ($P \leq 0.05$) longer lesions than the controls, but isolates ACT1, NSW1 and VIC2 did not spread tangentially larger than the controls seven months after inoculation (Fig. 3.3.1-4).

Internal discolouration was strongly correlated ($r = 0.97$, $P < 0.001$) with the external lesion lengths (data not presented).

Maximum lesion length (73 mm) and associated internal discolouration (110 mm) with WA1 (Fig. 3.3.1-8*d*) were significantly different to those produced by all other isolates including TAS1 (which was the isolate most aggressive to *E. nitens* seedlings).

Isolates VIC1, WA2, TAS1, TAS3, TAS8, TAS9 and TAS11 also produced large lesions with mean lesion lengths more than 30 mm long and tangential spread of more than 140° (Fig. 3.3.1-3 & 3.3.1.1-4).

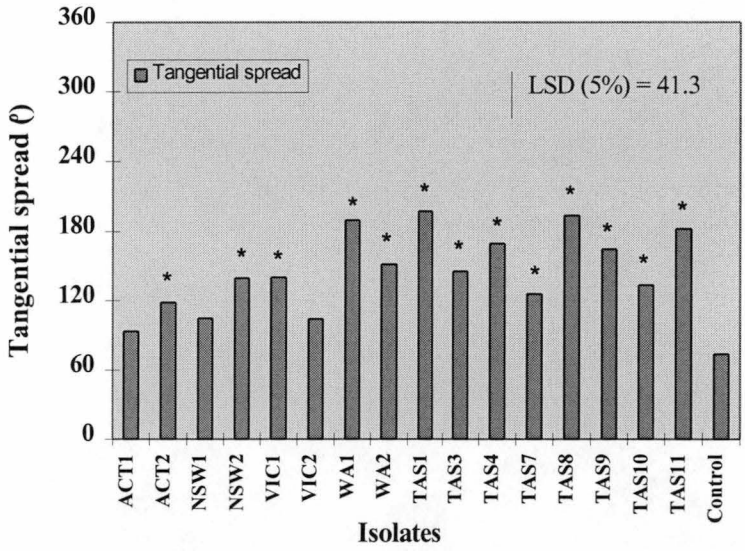


Fig. 3.3.1-4: Mean tangential spread of lesions on stems of *Eucalyptus globulus* seedlings inoculated with 16 isolates of *Endothia gyrosa*, assessed seven months after inoculation

Bars with an asterisk are significantly different to control.

One seedling of *E. globulus* inoculated with isolate TAS1 was dead seven months after inoculation.

Callus development was initiated 3 weeks after inoculation around all control and inoculation wounds. However, only control wounds and those inoculated with less pathogenic isolates (eg. ACT1) were fully occluded seven months after inoculation.

All the lesions produced by VIC1, WA1, WA2, TAS1, TAS8, TAS9 and TAS11 remained open and were covered with fruiting bodies seven months after inoculation.

All the isolates were successfully recovered from the lesion tissue with a high reisolation percentage ranging from 76% to 100% (Table 3.3.1-2).

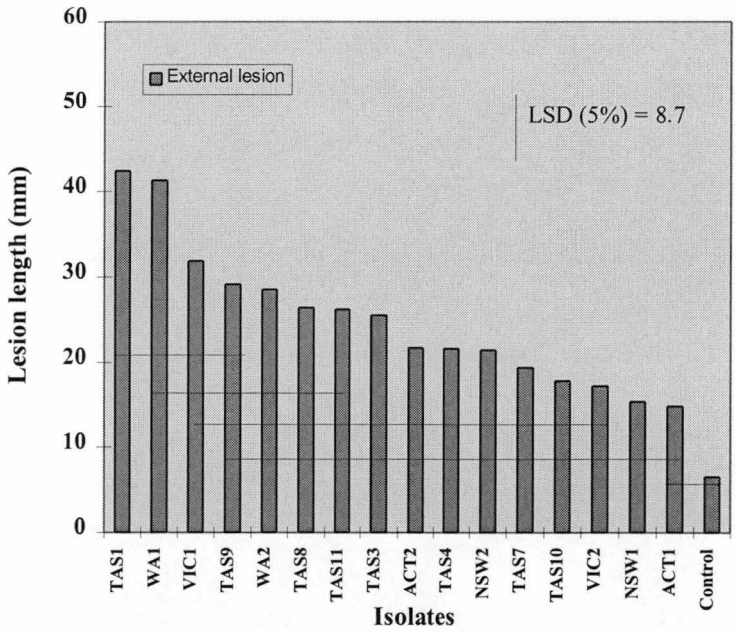


Fig 3.3.1-5: Overall mean length of external canker lesions on stems of 12-month-old seedlings of *Eucalyptus nitens* (NE-1) and *E. globulus* inoculated with 16 isolates of *Endothia gyrosa*, assessed seven months after inoculation

Bars sharing the same horizontal line are not significantly different.

3.3.1.3 Summary of seedling artificial inoculations

On the basis of the overall results expressed by the combined mean length of external lesions on seedlings of both *E. nitens* and *E. globulus*, no pathogenic variability in relation to isolate origin was found. For example, TAS1 from Tasmania, WA1 from Western Australia and VIC1 from Victoria were the most pathogenic isolates (Fig. 3.3.1-5). There were no significant differences in lesion lengths between 13 isolates (from TAS9 to ACT1 shown in Fig. 3.3.1-5).

For the two isolates from the each of the States and Territory, ACT, NSW, VIC and WA, no significant ($P>0.05$) differences in lesion lengths were found. (Fig. 3.3.1-5). For the eight isolates from Tasmania, TAS1 from Ridgley, northern Tasmania produced lesions significantly longer than those produced by all other isolates except TAS9 (which was also isolated from Ridgley).

When comparing the two eucalypt species, significant differences ($P<0.01$) in lesion size among the isolates can be observed (Appendix 6).

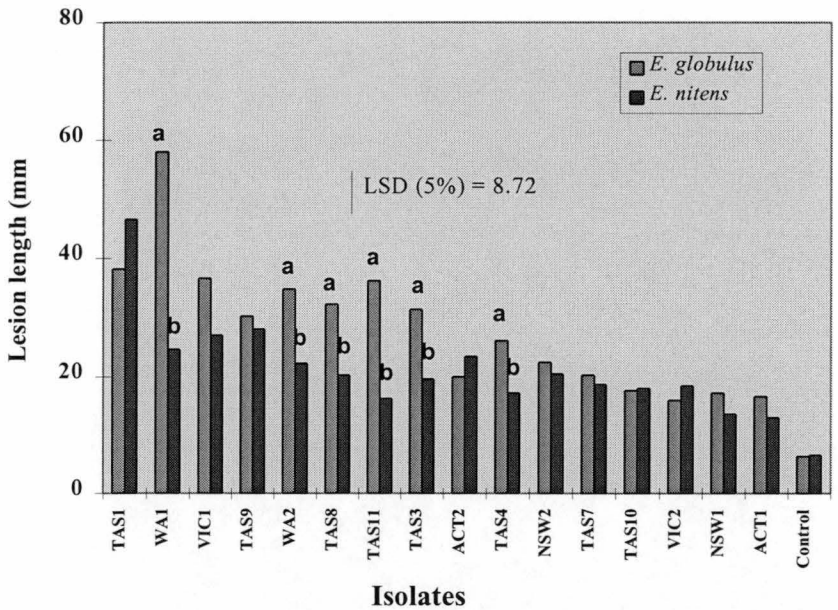
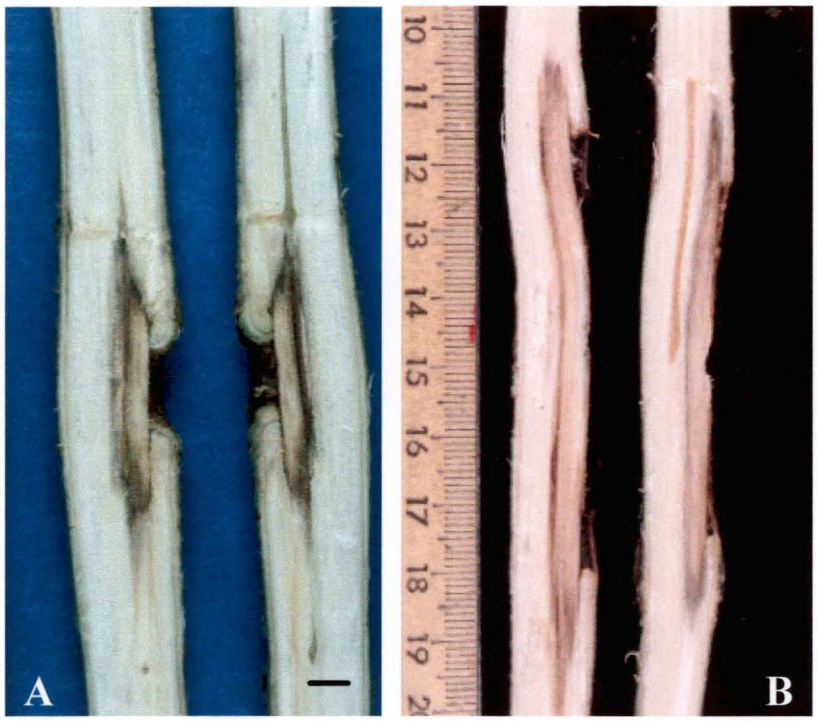


Fig. 3.3.1-6: Mean length (mm) of external canker lesions on *Eucalyptus nitens* and *E. globulus* seedlings inoculated with 16 isolates of *Endothia gyrosa*, seven months after inoculation

For each isolate bars in *E. globulus* and *E. nitens* with different letters are significantly different; for those not significantly different, letters are not given.



Bar = 4 mm for **A**

Fig. 3.3.1-7: Longitudinal sections of 12-month-old seedling stems of *Eucalyptus* spp. showing internal necrotic discolouration in sapwood due to inoculation of *Endothia gyrosa* seven months after inoculation.

A: discolouration produced by isolate WA1 in *E. nitens*;
B: discolouration produced by isolate WA1 in *E. globulus*

Bar = 6 mm for *A-D*

Fig. 3.3.1-8: Symptoms produced by 4 isolates of *Endothia gyrosa* on 12-month-old seedlings of *Eucalyptus globulus* seven months after inoculation, showing variations in lesion size between pairs of seedlings produced by the same isolate

A: lesions produced by isolate ACT2; **B**: lesions produced by isolate TAS10;
C: lesions produced by isolate NSW2; **D**: lesions produced by isolate VIC1

Eucalyptus globulus appeared to be more susceptible to the most pathogenic isolates than *E. nitens*. For example, lesions produced by WA1, WA2, TAS8, TAS11, TAS3 and TAS4 on *E. globulus* were significantly larger than those on *E. nitens* (Fig. 3.3.1-6). Internal discolouration associated with the lesions was also greater in *E. globulus*, as illustrated by WA1 in Fig. 3.3.1-7*a,b*.

Although lesions produced by TAS10, ACT2 and TAS1 were slightly smaller on *E. globulus* than on *E. nitens*, the difference was not significant (Fig. 3.3.1-6).

For both *E. nitens* and *E. globulus*, variations in lesion size in individuals within the seedlings inoculated with the same isolate were often observed (Fig. 3.3.1-8), although differences among replicates were not significant (Appendix 6).

3.3.2 INFECTION ABILITY OF CONIDIA AND ASCOSPORES

Ascospores and conidia were both able to produce lesions significantly different ($P \leq 0.05$) from controls when inoculated into wounds (Fig. 3.3.2-1). Lesions produced by conidia and ascospores were ellipsoid to fusoid in shape, dark brown to black (Fig. 3.3.2-2*a,b*). Four months after inoculation, the maximum mean lesion lengths produced by conidia and ascospores of TAS14 on NE-13 was respectively 12 mm and 14 mm. Tangential spread of the lesions produced by both ascospores and conidia was small (less than 95.3° and 104.7° respectively).

Internal discolouration by ascospores and conidia was highly correlated (r =0.89, $P<0.001$) with the external lesion length (Fig. 3.3.2-2*d,e*). The maximum lengths were 35 mm and 30 mm for ascospore and conidial inoculation respectively.

There were no significant differences between spore inoculation type (ascospore vs conidia) (Fig. 3.3.2-1). However, although not significantly different, lesions produced by ascospores tended to be longer than those produced by conidia. (Fig. 3.3.2-1).

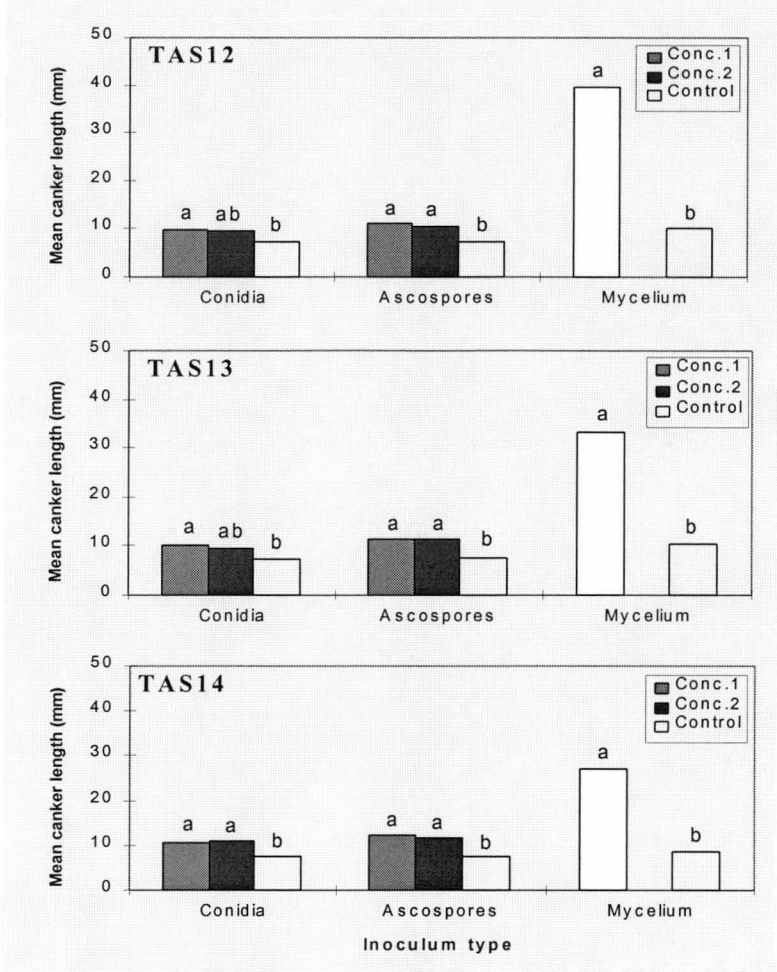


Fig. 3.3.2-1: Mean length of external lesions on seedling stems of *Eucalyptus nitens* inoculated with conidia, ascospores and mycelia of three *Endothia gyrosa* isolates, TAS12, TAS13 and TAS14; each bar represents the mean from 15 lesions (3 races x 5 replicates).

Bars with the same letter(s) are not significantly different within conidial and ascospore (LSD 5% = 2.68), and within mycelial (LSD 5% = 10.03) inoculum type.
Conc.1 = Concentration 1 (0.5 x 10^7 spores/ml for ascospores and 0.5 x 10^8 for conidia);
Conc.2 = Concentration 2 (2 x 10^7 for ascospores and 2 x 10^8 for conidia).
The same quantity of mycelium-mixed wheat/rice bran was used for all mycelial inoculations.

All lesions produced by mycelium were significantly longer ($P \leq 0.05$) than those produced by ascospores and conidia of the same isolates, spreading longitudinally far beyond inoculation points externally and internally (Fig. 3.3.2-2*c,f*). Tangential spread was up to 180° for most of the lesions. They were different to the lesions produced by conidia or ascospores in shape and size and were often sunken or flat at the centre with longitudinal cracks.

When harvested, 90% of the lesions (81 out of 90) produced by ascospores had completely occluded. No fruiting bodies were observed on the surface of any lesions whether occluded or not.

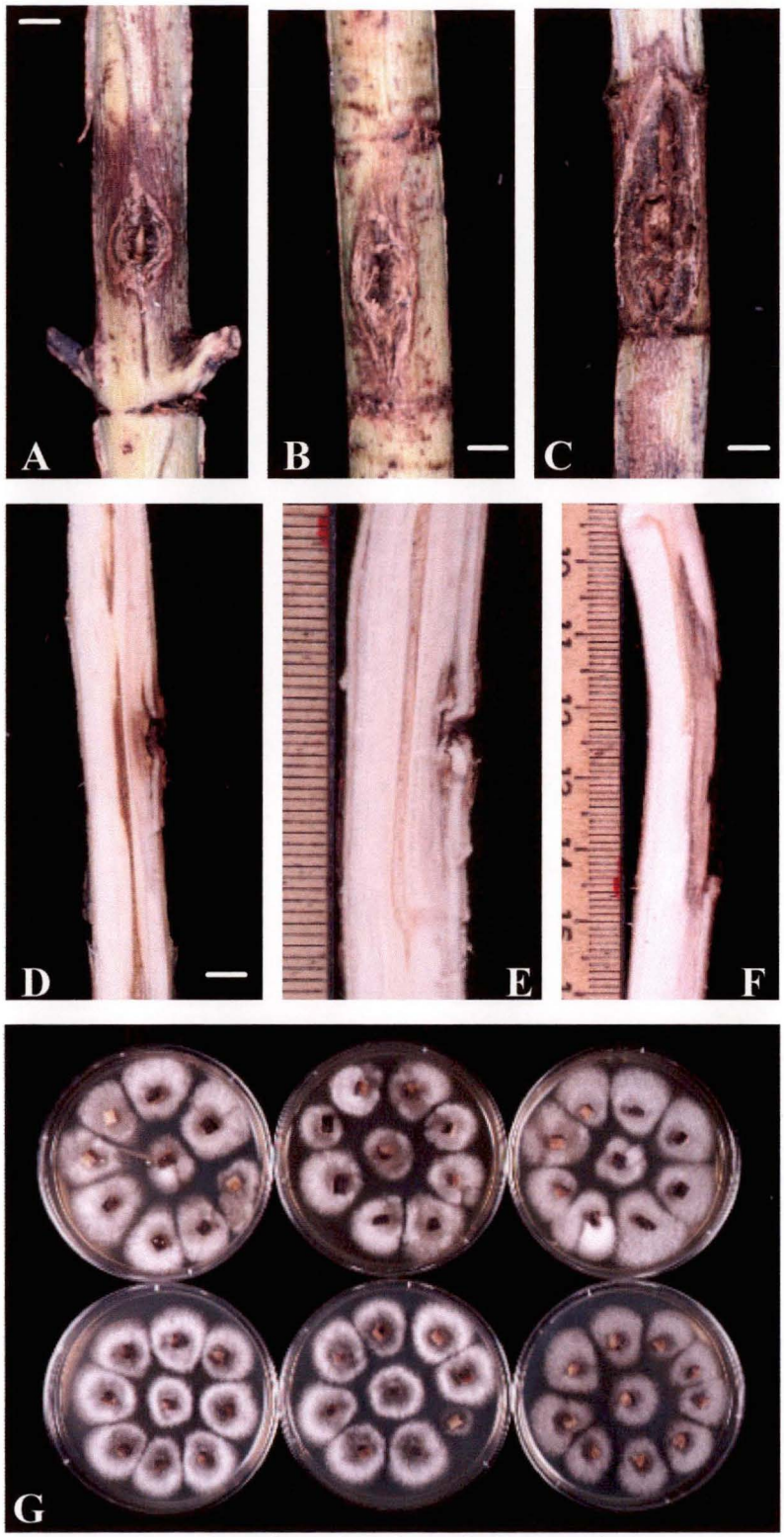
Only 62% of the lesions induced by conidia had occluded after 4 months. Twenty six percent of all lesions resulting from conidial infection, produced conidiomata. They all remained open at 4 months.

When harvested at 4 months, all lesions resulting from mycelial inoculation were still open and covered with abundant conidiomata, although callus tissue was observed around some of the lesions.

All attempts at re-isolating *E. gyrosa* from lesions induced by conidia or mycelium were successful, with a 100% of fungal recovery (Fig. 3.3.2-2*g*). Reisolations from lesions induced by ascospores were only slightly less successful (90%). Cultures of stem pieces excised from the margins of lesions originating from either ascospores or conidia produced conidiomata after two weeks.

The two different spore concentrations used for each ascospore and conidial inoculum both resulted in infection. Lesions produced with the more concentrated spore suspension were slightly longer than those resulting from the lower concentration of spores, although there were no significant differences ($P>0.05$) in lesion length between the two concentrations within each spore type.

Significant differences in lesion length were found between the *E. nitens* seedlings from different localities within spore ($P\leq0.01$) and mycelial ($P\leq0.05$) inoculation type.



Bar = 4 mm for **A** & **C**; 3 mm for **B;** 5 mm for **D**

Fig. 3.3.2-2: Inoculation of 17-months-old seedlings of *Eucalyptus nitens* with conidia, ascospores and mycelia of three *Endothia gyrosa* isolates

A-C. lesions produced by conidia (A), ascospores (B) and mycelium (C) four months after inoculation;

D-F. internal discolouration of wood associated with inoculation of conidia (D), ascospores (E) and mycelium (F);

G. reisolation of *E. gyrosa* from lesions inoculated with conidia (left pair of plates); ascospores (middle pair) and mycelium (right pair)

3.3.3 COLONY MORPHOLOGY, FUNGICIDE RESPONSE AND VEGETATIVE COMPATIBILITY GROUPS

3.3.3.1 Colony morphology

A dendrogram of the 133 isolates constructed using cluster analysis is shown in Fig. 3.3.3-1. Four types of colony morphologies were observed among 133 isolates of *E. gyrosa* when grown on MEA (Fig. 3.3.3-2).

The most common colony habit was type I (with fluffy, white or grey aerial mycelium) which was found among 93 isolates. The majority (83) of these isolates originated from Tasmania. Within the group most of the isolates sporulated abundantly but a few (eg. TAS4) seldom sporulated.

The second predominant colony was type II (with thin, vitelline or orange-coloured to reddish brown appressed mycelium). Twenty four isolates were found associated with this colour type. Of the 24 isolates, 13 were from other states of Australia, eg. ACT (one isolate), NSW (three isolates), Victoria (five isolates) and WA (four isolates). Only 8 isolates were from Tasmania. The two Italian isolates and one South African isolate (CRY287) also fell into this group.

Five isolates, ATCC48192, CBS508.76 and CBS509.76 from USA and two from South Africa (CRY57, CRY94) were found in type III (with dense to fluffy, buff or vitelline to orange-coloured aerial mycelium).

The remaining 12 isolates, all from Australia belonged to type IV (with thin or plain, white to grey appressed mycelium). This type was closer to type I than were type II and III and fell into one major cluster with type I (Fig. 3.3.3-1).

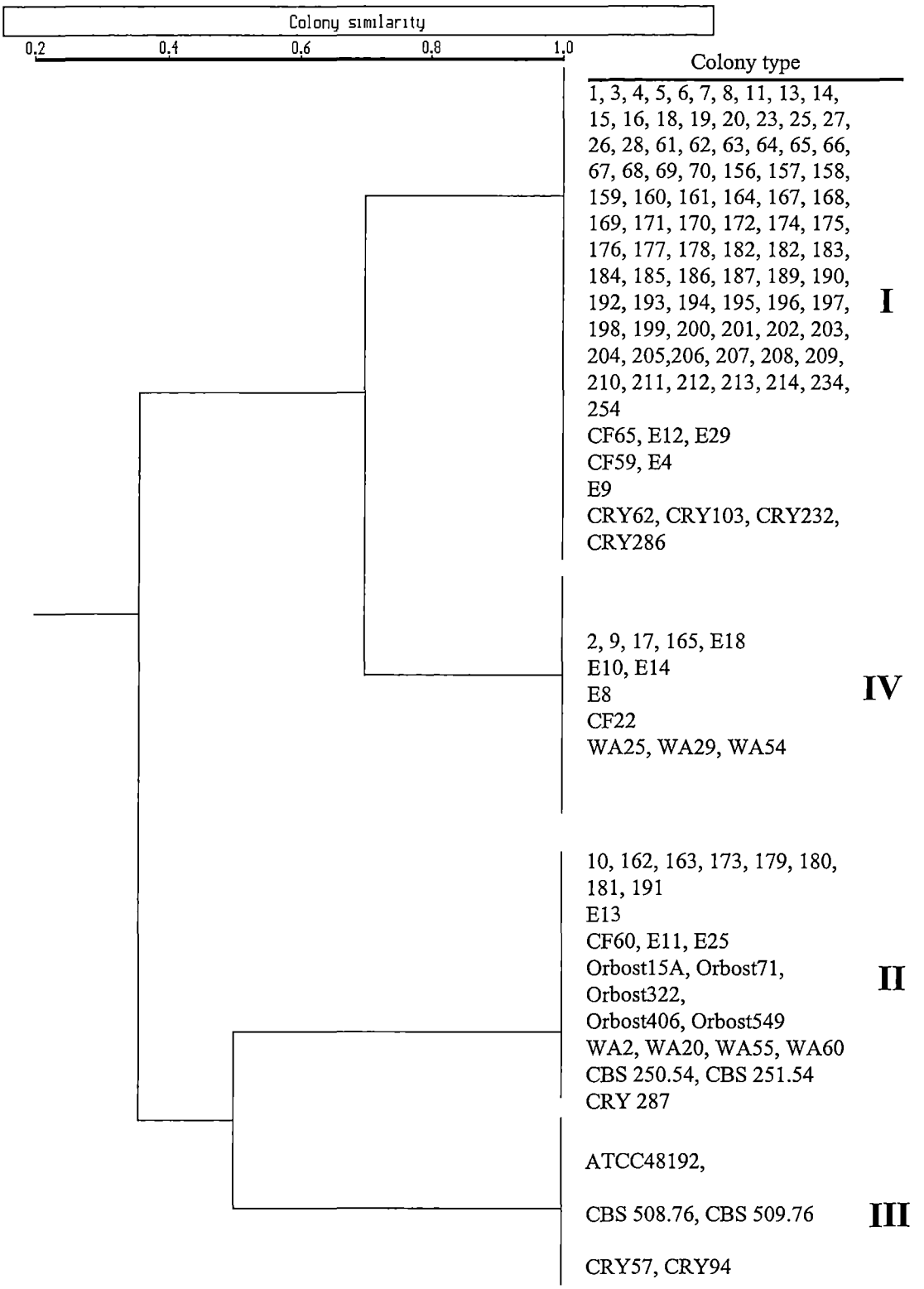


Fig. 3.3.3-1: Dendrogram produced by colony similarity (data) using UPGMA cluster analysis showing relationship among 133 isolates of *Endothia gyrosa*.

All isolates numbered without letter(s) and E18 are collected from Tasmania; CF65, E10, E12, E13, E14 and E29 from Australian Capital Territory; CF59, CF60, E4, E8, E11 and E25 from New South Wales; CF22, E9, Orbost15a, 71, 322, 406 and 549 from Victoria; All isolates with WA designation from Western Australia; CBS 250.54 and CBS 251.54 from Italy; ATCC48192, CBS508.76 and CBS509.76 from the United States of America and all the isolates with CRY designation from South Africa.

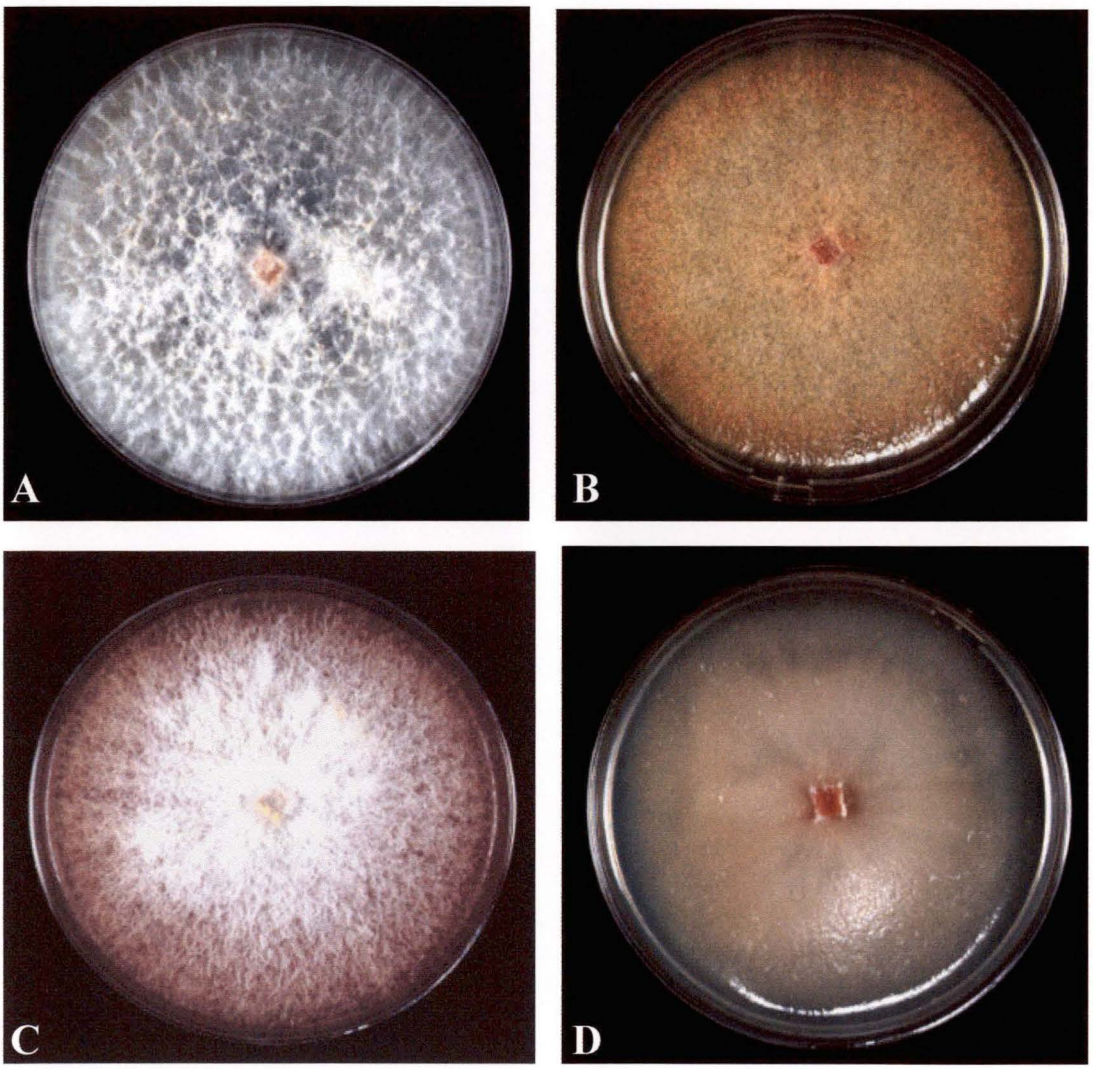


Fig. 3.3.3-2: Examples of *Endothia gyrosa* colony types on malt extract agar (MEA) after 20 days

A. Type I (TAS1);
B. Type II (ITA1);
C. Type III (USA2);
D. Type IV (ACT1)

No changes of colony types of cultures for isolates in Table 3.2-4 were observed when they were exposed to light and subcultured for several times under the same incubation conditions.

3.3.3.2 Response to fungicides

Only data from cholorothalonil are presented as the growth of nearly all isolates tested on both benomyl and thiabendazole was inhibited at a concentration of 50 mg/L.

Three groups were determined based on the radial growth of the isolates to cholorothalonil (Table 3.3.3-2). Isolates from South Africa and Italy, as well as all but one Tasmanian isolate (TAS1-TAS5) were grouped as S. They were sensitive to cholorothalonil at 50 mg/L with mean radial growth (as a percentage of control) ranging from 13.9 -19.3%. Two American isolates, along with the isolates from ACT, NSW and VIC were grouped as SR as they appeared slightly resistant with mean radial growth ranging from 20.9-26.9% (Table 3.3.3-2). The Western Australian isolates and one Tasmanian isolate (TAS6), grouped as MR were moderately resistant (41.4-53.1% of control growth) to cholorothalonil.

No isolates were classified as highly resistant (60-80% of control growth) to any concentration of cholorothalonil evaluated.

3.3.3.3 Determination of VC-groups

On 2% MEA, pairs of *E. gyrosa* did not show clear incompatibility reactions. Most incompatible pairs showed a weak dark line (Fig. 3.3.3-3). Strong vegetative incompatibility reactions were only shown by the isolates from overseas (for instance, ITA1) when paired with others. Dark barrage lines separated these colonies as illustrated in Fig. 3.3.3-3 & Fig. 3.3.3-4a.

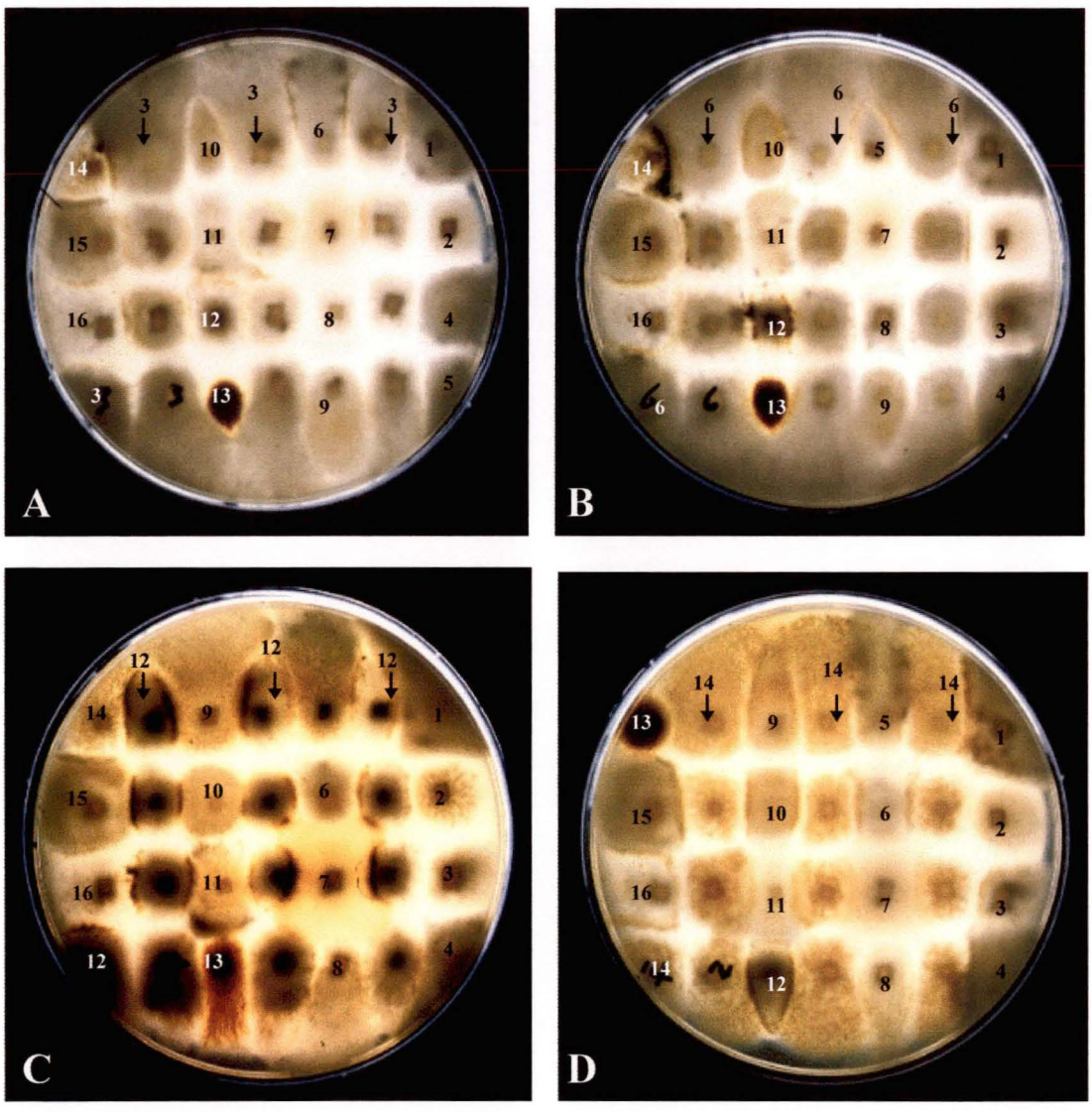


Fig. 3.3.3-3: Four examples of vegetative compatibility (VC) group tests among sixteen *Endothia gyrosa* isolates

A. Isolate No. 3 (TAS3) (arrow column);
B. Isolate No. 6 (TAS6);
C. Isolate No. 12 (USA1);
D. No. 14 (ITA1) paired with other 15 isolates and with itself.

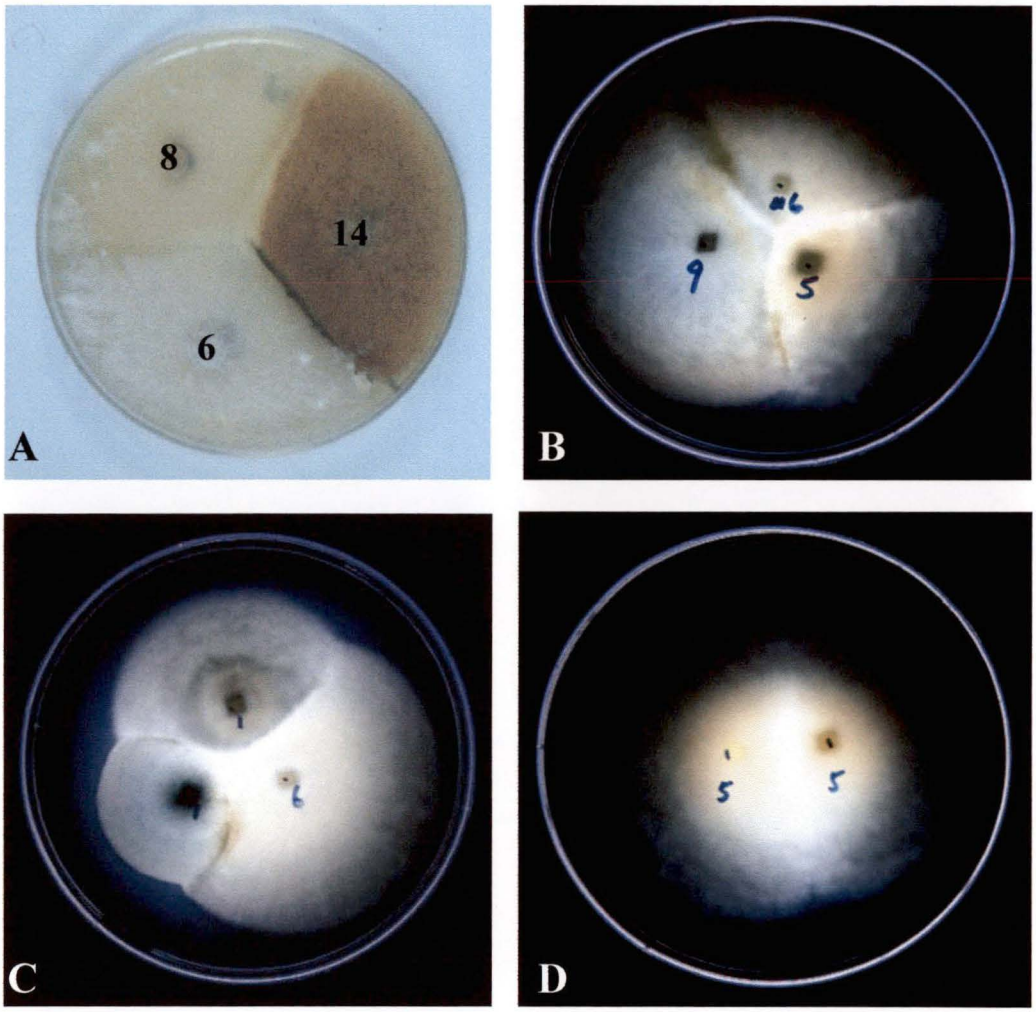


Fig. 3.3.3-4: Vegetative compatibility (VC) reactions between *Endothia gyrosa* isolates on 2% malt extract agar (A) and medium containing a pH indicator (B-D)

A. Three incompatible isolates on 2% malt extract agar; No. 8 (NSW1), No. 6 (TAS6) and No. 14 (ITA1) showing a weak incompatibility reaction line between No. 8 and No. 14 and a dark barrage line between No. 14 and No. 6;

B. Isolates No. 6 (TAS6), No. 9 (VIC1) and No. 5 (TAS5) showing green incompatible reaction lines between each pairing isolates on medium containing a pH indicator;

C. Pairing between isolates No. 1 (TAS1), No. 7 (ACT1) and No. 6 (TAS6) on the pH indicator medium;

D. Self-pairing of isolate No. 5 (TAS5) showing no green line between two colonies on pH indicator medium

Table 3.3.3-1: Vegetative incompatibility reactions between 16 isolates of *Endothia gyrosa* Minus sign (-) indicates vegetative incompatibility; Plus sign (+) indicates vegetative compatibility

	TAS1	TAS2	TAS3	TAS4	TAS5	TAS6	ACT1	NSW1	VIC1	WA1	WA2	USA1	USA2	ITA1	SA1	SA2
TAS2	-															
TAS3	-	+														
TAS4	-	+	+													
TAS5	-	+	+	+												
TAS6	-	-	-	-	-											
ACT1	-	-	-	-	-	-										
NSW1	-	+	+	+	+	-	-									
VIC1	-	-	-	-	-	-	-	-								
WA1	-	-	-	-	-	-	-	-	+							
WA2	-	-	-	-	-	-	-	-	+	+						
USA1	-	-	-	-	-	-	-	-	-	-	-					
USA2	-	-	-	-	-	-	-	-	-	-	-	+				
ITA1	-	-	-	-	-	-	-	-	-	-	-	-	-			
SA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
SA2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

On PDAvc medium containing a pH indicator, a green line between pairing isolates made it possible to distinguish between VC groups. Most of the incompatible pairings produced a visible green line between the colonies (Fig. 3.3.3-4b, c) and all self pairings were vegetatively compatible without any reaction lines visible (Fig. 3.3.3-4d).

Depending on pairing isolates, several to many pycnidia were often observed forming along one side of colony for most of the incompatible pairings.

A total of nine VC groups were identified among the 16 isolates tested (Table 3.3.3-1 & Table 3.3.3-2). Eleven Australian isolates were found belonging to five groups, while the other five overseas isolates to four different groups.

Four isolates from Tasmania, TAS2 through TAS5 and one from New South Wales (NSW1) were found to belong to group II. Three isolates, one from Victoria (VIC1) and two from Western Australia (WA1, WA2) belonged to group V and two isolates

from USA (USA1, USA2) to group VI. The remaining 6 isolates, TAS1, TAS6, ACT1, ITA1, SA1 and SA2 each represented single-member groups.

Vegetative compatibility was also confirmed by checking under light microscopy for anastomoses forming between isolates assessed as compatible. An example of a line drawing is given in Fig. 3.3.3-5 to illustrate 2 day-old compatible anastomosis between two Tasmanian isolates TAS4 and TAS5. Similar compatible anastomosis between other vegetative compatible isolates were also observed.

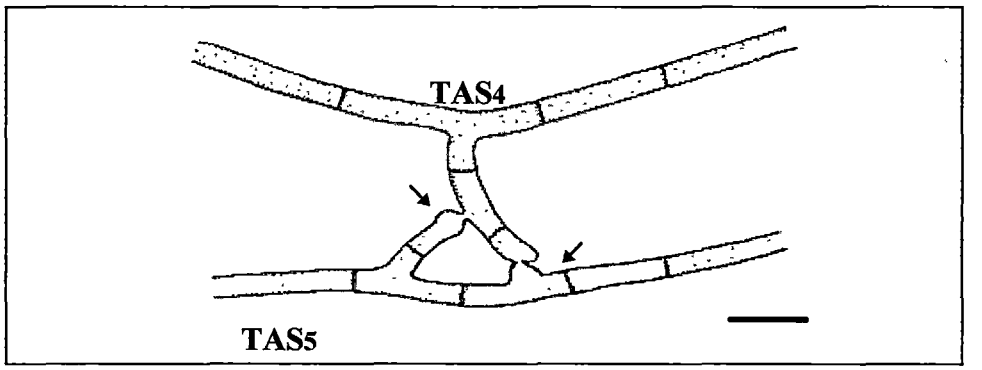


Fig. 3.3.3-5: A line drawing illustrating 2 day-old compatible anastomosis (arrows) between two Tasmanian isolates of *E. gyrosa*, TAS4 and TAS5 (Bar = 15 μm).

3.3.3.4 Summary for Section 3.3.3

Four types of colonies were classified among 133 isolates based on colony features. Among 16 isolates selected, only three groups were found based on their resistance to cholorothalonil but nine VC groups were determined (Table 3.3.3-2).

Table 3.3.3-2. Colony type, response to fungicide (radial growth percentage of control), vegetative compatibility (VC) groupings for 16 *Endothia gyrosa* isolates

Isolate	Colony type[A]	Response to fungicide[B]	VC group
TAS1	I	S	I
TAS2	IV	S	II
TAS3	I	S	II
TAS4	I	S	II
TAS5	I	S	II
TAS6	I	MR	III
ACT1	IV	SR	IV
NSW1	IV	SR	II
VIC1	II	SR	V
WA1	II	MR	V
WA2	II	MR	V
USA1	III	SR	VI
USA2	III	SR	VI
ITA1	II	S	VII
SA1	I	S	VIII
SA2	III	S	IX

[A]Colony types:
I = fluffy, white or grey aerial mycelium;
II = with thin, vitelline or orange-coloured to reddish brown appressed mycelium;
III = with dense to fluffy, buff or vitelline to orange-coloured aerial mycelium;
IV = with thin or plain, white to grey appressed mycelium
[B]S = sensitive; SR = slightly resistant; MR = moderately resistant.

3.3.4 DNA POLYMORPHISM

3.3.4.1 RFLP of the ITS region

The amplified DNA corresponding to the ITS yielded four polymorphism groups having fragments very close in size (Fig.3.3.4-1).

The first group (fragment size 640bp) included USA1, USA2, and SA2.

The second group (fragment size 660bp) included TAS1, TAS6, ACT1, VIC1, WA1, WA2 and SA1.

Isolates TAS2, TAS3, TAS4, TAS5 and NSW1 having fragments of 670bp in size belonged to the third group.

The fourth group included only ITA1 with a fragment of 680bp.

Restriction endonucleases *Cla* I, *Dpn* II, *Hae* III and *Hin*fI all appeared to cut at the same restriction sites (Table 3.3.4-1 & Fig. 3.3.4-1), although very slight differences in banding patterns were observed because of fragment size polymorphisms.

With digestion by *Cla* I, for example, banding patterns were observed as follows:

The Australian isolates fell into two groups: group I including TAS1, TAS6, ACT1, VIC1, WA1, and WA2 group II including TAS2, TAS3, TAS4, TAS5 and NSW1.

The two American isolates (USA1 and USA2) belonged to group III and the Italian isolate represented a single-member group, group IV.

Table 3.3.4-1: Approximate size of restriction fragments (rounded to the nearest 10 bp) following digestion with four restriction enzymes of PCR-amplified internal transcribed spacer (ITS) region from 16 isolates of *Endothia gyrosa*

	Australian isolates											Overseas isolates				
	TAS1	TAS2	TAS3	TAS4	TAS5	TAS6	ACT1	NSW1	VIC1	WA1	WA2	UAS1	UAS2	ITA1	SA1	SA2
Uncut	660	670	670	670	670	660	660	670	660	660	660	640	640	680	660	640
***Cla* I**	360	360	360	360	360	360	360	360	360	360	360	360	360	360	360	360
	300	310	310	310	310	300	300	310	300	300	300	280	280	320	300	280
***Dpn* II**	310	310	310	310	310	310	310	310	310	310	310	310	310	320	310	310
	250	260	260	260	260	250	250	260	250	250	250	220	220	300	250	220
	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	50[a]	60	50[a]	50[a]
***Hin* fI**	250	260	260	260	260	250	250	260	250	250	250	210	210	270	250	210
	160	160	160	160	160	160	160	160	160	160	160	160	160	160	160	160
	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]
	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
***Hae* III**	280	280	280	280	280	280	280	280	280	280	280	280	280	280	280	280
	190[a]	200	200	200	200	190[a]	190[a]	200	190[a]	190[a]	190[a]	180[a]	180[a]	210	190[a]	180[a]
		190	190	190	190			190						190		

[a]Two restriction fragments have the same apparent size.

The two South African isolates fell into two different groups with SA1 having similar band patterns to the Australian isolates of group I and SA2 having the same band patterns as American isolates in group III.

3.3.4.2 RAPD with nuclear DNA

Most RAPD amplifications were repeated twice and both faint and intense bands were scored if shown to be reproducible in separate runs. The size of the DNA fragments used for evaluation ranged approximately from 0.21 kbp to 1.2 kbp. A total of 164 RAPD markers scored with 13 primers were used for evaluation. RAPD markers for all 16 isolates generated by primers, UBC-81, UBC-215, UBC-218 and OP-B08 are shown in Fig. 3.3.4-2 and Table 3.3.4-2. These show several types of banding patterns were consistently observed with each of the 13 primers.

A similarity matrix was generated with the RAPD markers (Table 3.3.4-3). The *E. gyrosa* isolates were divided into 10 groupings [all isolates belonging to the same group have bandsharing similarity coefficients (BSC) above 80%] (Fig. 3.3.4-3).

Three isolates were found to belong to group IV (VIC1, WA1, WA2), 4 isolates to group V (TAS2, TAS3, TAS4, TAS5), 2 isolates to group IX (USA1, USA2) and the remaining 7 isolates each represented single-member groups, I (TAS1), II (ACT1), III (TAS6), VI (NSW1), VII (SA1),VIII (SA2) and X (ITA1).

At least one single DNA fragment (mostly ca. 1 kbp) was amplified from all isolates by 10 of the 13 arbitrary primers used in the study, showing a certain relationship among the tested isolates.

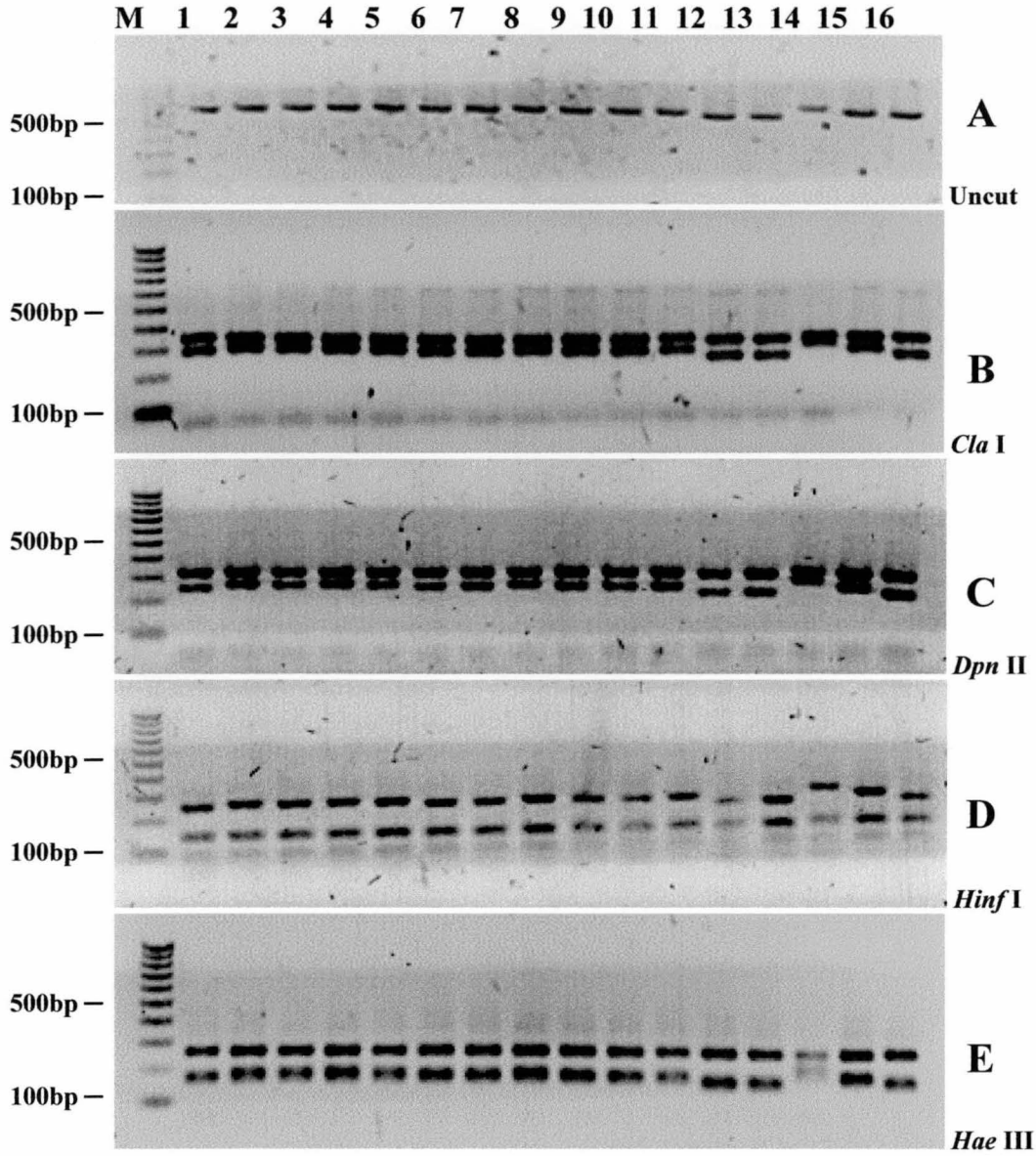


Fig. 3.3.4-1: Gel electrophoresis: (A) PCR amplified ITS region of rDNA (with primers ITS1 and ITS4); (B-E) restriction endonuclease digestion of ITS region with *Cla* I; *Dpn* I; *Hinf* I and *Hae* III respectively

Lanes 1 to 11: Australian isolates (TAS1, TAS2, TAS3, TAS4, TAS5, TAS6, ACT1, NSW1, VIC1, WA1 and WA2);

lanes 12 and 13 : American isolates (USA1 and USA2);

lane 14: Italian isolate (ITA1);

lanes 15 and 16: South African isolates (SA1 and SA2)

lane M: molecular weight markers (Bresatec DMW-100L) 100-bp DNA ladder.

Table 3.3.4-2. Molecular weights (base pairs) of DNA bands in *Endothia gyrosa* populations identified as 10 different RAPD phenotypes (primer UBC-81). Presence (1) or absence (0) of bands

	RAPD phenotypes detected with primer UBC-81															
DNA band	I	V				III	II	VI	IV			IX		X	VII	VIII
(bp)	TAS1	TAS2	TAS3	TAS4	TAS5	TAS6	ACT1	NSW1	VIC1	WA1	WA2	USA1	USA2	ITA1	SA1	SA2
1200	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1180	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	1
1150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
1140	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1
1100	0	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1
1080	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1020	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
950	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	1
800	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1
650	1	0	0	0	0	1	1	0	1	1	1	0	0	1	1	0
510	1	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0
400	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
380	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0
300	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0

Table 3.3.4-3. Similarity matrix of *Endothia gyrosa* based on RAPD data using Simple Matching Coefficients

	TAS1	TAS2	TAS3	TAS4	TAS5	TAS6	ACT1	NSW1	VIC1	WA1	WA2	USA1	USA2	ITA1	SA1	SA2
TAS1	1.00															
TAS2	0.598	1.00														
TAS3	0.598	1.000	1 00													
TAS4	0 591	0.994	0.994	1.00												
TAS5	0 604	0.994	0 994	0 988	1.00											
TAS6	0 762	0.640	0.640	0.646	0.634	1.00										
ACT1	0 787	0 628	0.628	0.634	0.634	0.756	1.00									
NSW1	0.720	0.768	0 768	0 762	0 774	0 762	0 774	1 00								
VIC1	0 713	0.579	0 579	0.573	0 585	0 695	0 707	0 665	1 00							
WA1	0.695	0.573	0 573	0 567	0 579	0 677	0 701	0 646	0 982	1 00						
WA2	0 701	0.567	0 567	0 561	0 573	0 683	0 707	0 652	0 988	0 994	1.00					
USA1	0 470	0 470	0 470	0 463	0 463	0 488	0 427	0 457	0 415	0 421	0 415	1.00				
USA2	0 457	0.470	0.470	0 463	0 463	0 488	0 439	0 470	0.427	0.433	0.427	0.976	1.00			
ITA1	0 494	0.494	0.494	0.500	0.488	0.512	0.500	0.518	0.427	0.433	0.427	0.659	0.634	1.00		
SA1	0 567	0 616	0.616	0.610	0 610	0.598	0.585	0 665	0 585	0.579	0.585	0.463	0.451	0.573	1.00	
SA2	0 482	0.506	0.506	0 512	0.500	0 488	0 512	0.543	0 488	0 494	0.500	0 561	0 549	0 659	0.732	1.00

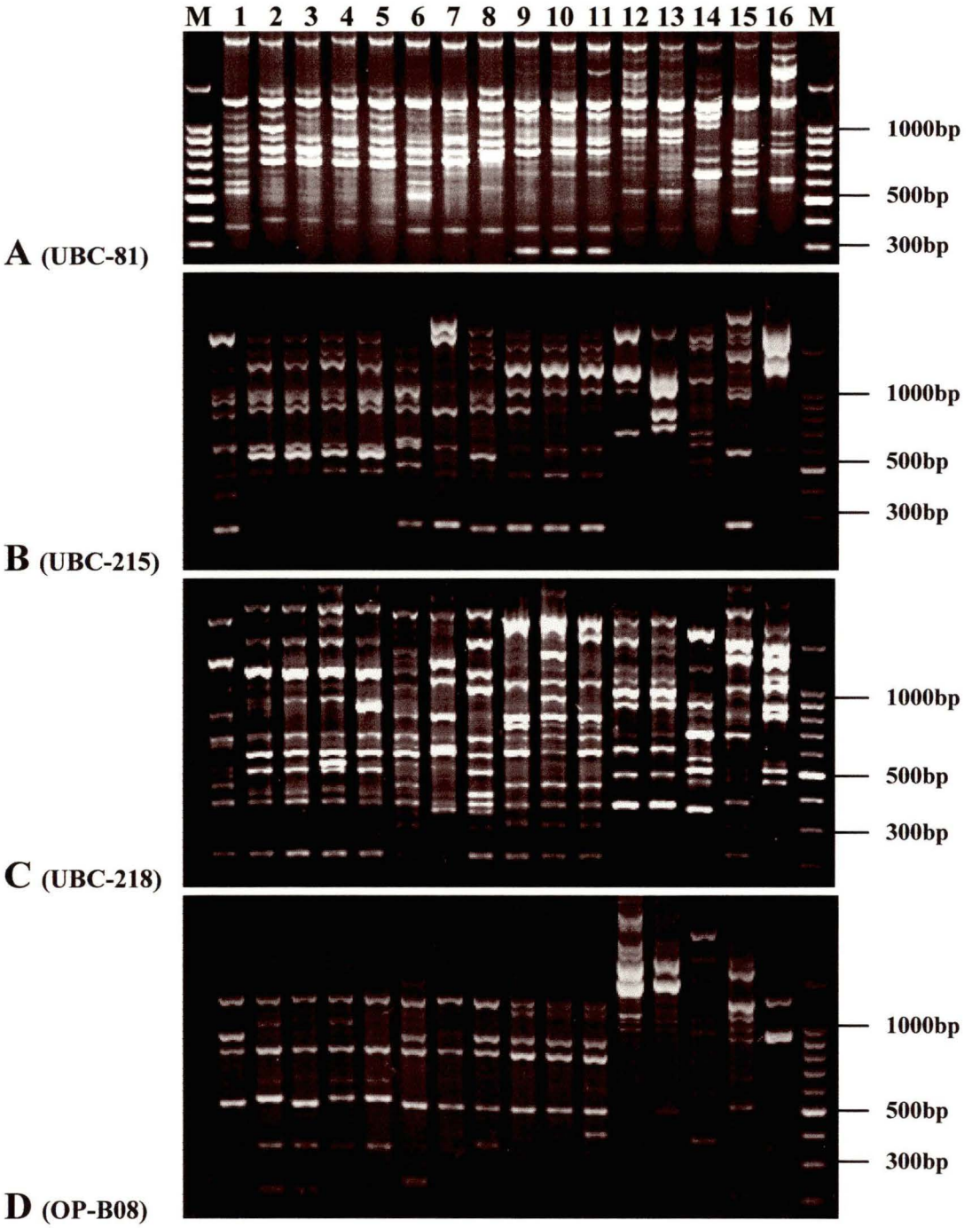


Fig. 3.3.4-2: Gel electrophoresis of PCR-RAPD products for nuclear DNA of sixteen isolates of *Endothia gyrosa*.

A: primer UBC-81;
B: primer UBC-215;
C: primer UBC-218;
D: primer OP-B08.
Lanes 1 to 11: Australian isolates (TAS1, TAS2, TAS3, TAS4, TAS5, TAS6, ACT1, NSW1, VIC1, WA1 and WA2);
lanes 12 and 13: American isolates (USA1 and USA2);
lane 14: Italian isolate (ITA1); lanes 15 and 16: South African isolates (SA1 and SA2);
lane M: molecular weight markers (Promega, Madison USA) 100-bp DNA ladder.

Figure 3.3.4-3 shows that all the 16 isolates fell into three discrete clusters (Branches 1 and 2). Branch 2 separated five overseas and eleven Australian isolates into two clusters with a mean bandsharing similarity coefficients of 54% at the breaking point. Within the isolates from overseas, two isolates from South Africa appeared to be more closely related to the Australian isolates than those from the north hemisphere (USA and Italy).

Australian isolates fell into two discrete clusters (branch 3) (Fig. 3.3.4-3). One New South Wales isolate (NSW1) and four Tasmanian isolates, three from Tewkesbury (TAS2-4), one from Camden (TAS5) were fell into one cluster. Within this cluster, the four Tasmanian isolates were narrowly grouped into one RAPD group (V) (mean BSC 99.3%).

The other cluster included two Tasmanian isolates, (TAS1 and TAS6 respectively from Tewkesbury and Camden), two Western Australian isolates (WA1, WA2) and one Victorian isolate (VIC1). The latter three isolates (WA1, WA2 and VIC1) also formed a narrowly related RAPD group (IV) (mean BSC 98.8%) within this cluster.

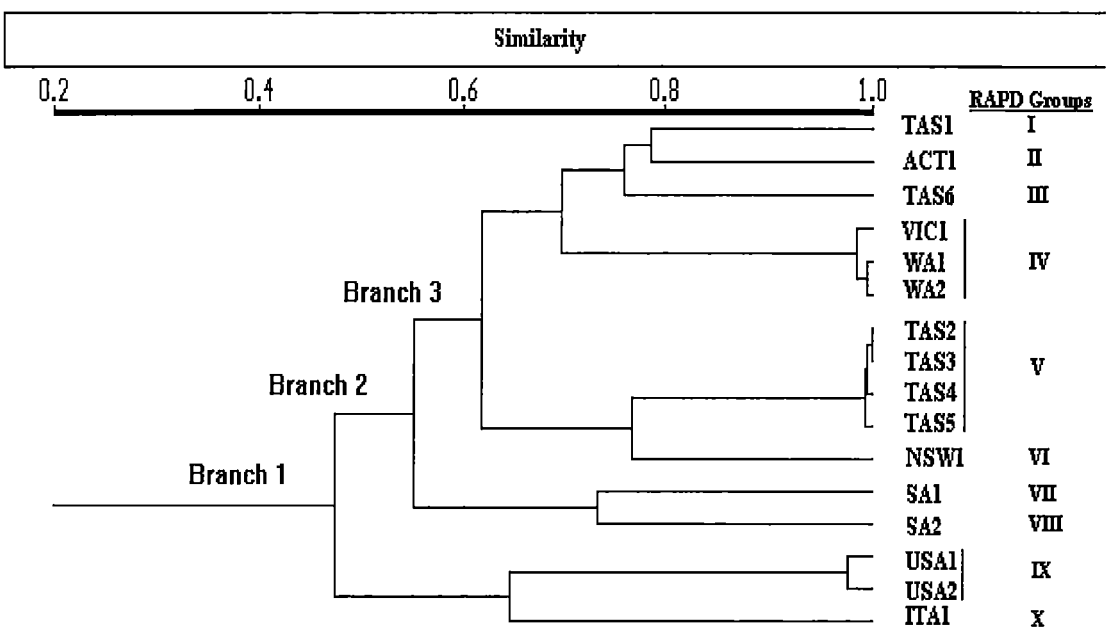


Fig. 3.3.4-3. Dendrogram produced by RAPD data (similarity matrix) using UPGMA cluster analysis showing relationship among 16 isolates of *Endothia gyrosa*.

3.4 DISCUSSION

INTRASPECIFIC VARIATION IN PATHOGENICITY

Observed level of intraspecific variation

All the *E. gyrosa* isolates, with the exception of ACT1, caused external lesions that were significantly larger than the lesions of the control treatment. Although the experimental data could have been improved by using single provenance or clonal plants the three isolates, TAS1, TAS3 and TAS9 tested on both seedlings and trees in Chapter 2 showed the same trends in their ability to cause lesions in this separate trial, indicating that the observed pathogenic variability in the isolates was consistent.

While there was variation between isolates in their pathogenicity, as expressed by lesion length and tangential spread, only two isolates produced significantly ($P<0.05$) larger lesions than the others. These isolates were TAS1 from Tasmania inoculated on *E. nitens* and WA1 from Western Australia on *E. globulus*. Hence it appeared that intraspecific variation within the *E. gyrosa* isolates was relatively small. This finding is supported by the work of Old *et al.* (1990) who demonstrated small variations in the pathogenicity of five isolates of *E. gyrosa* inoculated on a range of eucalypt species.

The pathogenicity of the isolates does not appear to be linked to their geographical origin. The three most pathogenic isolates, TAS1, WA1 and VIC1 were from three geographically distinct regions of Australia, that of Tasmania, Western Australia and Victoria respectively. While there were no significant differences in the average pathogenicity of isolates from each different region, most of the Tasmanian isolates (TAS1, TAS3, TAS8, TAS9 and TAS11) tended to be more aggressive than those from southeast Australia (ACT and NSW).

Within geographical regions, only those isolates from Tasmania exhibited significant ($P<0.05$) intraspecific variation. Old *et al.* (1990) did demonstrate similar levels of variation *E. gyrosa* pathogenicity with isolates from southeast Australia (ACT and

NSW). In the present study intraspecific variation in regions other than Tasmania could not be statistically quantified as there were too few tested for each region.

There did not appear to be any association in pathogenicity levels with the host origin of isolates, eg. isolates from *E. maculata* (WA1), *E. regnans* (TAS11), *E. sieberi* (VIC1), *E. viminalis* (ACT2) and *E. wandoo* (WA2) were pathogenic to both *E. nitens* and *E. globulus*. Similar results have been reported by Old *et al.* (1990).

Influence of culture age on fungal pathogenicity to plants has been found for many other pathogens. Hodges (1972), for example, observed that isolates of *Helminthosporium sorokinianum* from older cultures were less pathogenic to *Poa pratensis* than those from younger cultures. Abd-Elrazik *et al.* (1978) attributed a low pathogenicity of virulent isolates of *Cephalosporium maydis* to maize to an increase of some enzyme production, such polyphenol oxidase, peroxidase cytochrome oxidase and beta-glucosidase with culture age, as highly pathogenic isolates showed lower activity of these enzymes. Holmes (1973) tested pathogenicity of 24 eight-year-old *Ceratocystis ulmi* Buisman isolates on *Ulmus hollandica* seedings in greenhouse and found that only 7 out of 24 caused wilt symptoms. It was concluded that old cultures should not be used to test the resistance of elms to *C. ulmi*.

The tendency of the Tasmanian isolates to be more pathogenic could be attributed to the younger age of the cultures. All the isolates from mainland Australia were at least 10 years old whereas 7 of the 8 Tasmanian isolates were isolated in 1995. However greater pathogenicity of younger isolates was not consistent. Isolates WA1, WA2 and TAS11 (in culture for 10 years) were comparatively pathogenic.

Factors influencing the expression of pathogencity by a culture are probably subject to more complex influences than age alone. Different isolates may vary in regard to their response to storage conditions (which can vary greatly between laboratories and culture collections). Subculturing or repeat passage through host tissue may select for more "culture-friendly" and less pathogenic isolates.

Influence of host response on intraspecific variation

Although length of induced lesion on seedlings is regarded as a reliable indicator of virulence in artificial inoculations, the level of seedling mortality is also important in pathogenicity assessment. In artificial inoculation trials with extremely virulent pathogens such as *Cryphonectria parasitica* the rate of mortality is very much higher (Griffin *et al.* 1978, 1983). Seedling mortality was zero or very low for all *E. gyrosa* isolates in this study and others (Davison and Tay 1983; Old *et al.* 1986, 1990; van der Westhuizen *et al.* 1993). Yet in Western Australia *E. gyrosa* isolates are currently causing high levels of mortality to 1-year-old *E. globulus* in artificial inoculation trials (Shedley 1998, pers. comm.). What are the reasons for this mortality? Why is the same host species at the same age as in the present study more susceptible to *E. gyrosa*? Are the West Australian isolates more virulent? Is there greater intraspecific variation in *E. gyrosa* pathogenicity than detected in this study?

The author suggests that levels of virulence expressed by this opportunistic fungus in nature will be highly unpredictable, subject to the multitude of influences governing host response such as vigour, host species, provenance and management practices. Although it is difficult to make comparisons with results from experiments under different conditions, especially if conducted many years ago, evidence for the latter statement is provided by comparing the present study with others:

- host species and expressed virulence

On 12 month old seedlings of four eucalypt species (*E. grandis*, *E. maculata*, *E. regnans* and *E. saligna*) Old *et al* (1990) recorded that the longest canker among lesions produced by five *E. gyrosa* isolates was only small, 13.6 mm three months after inoculation. The highest mean lesion length of 10 mm was found on *E. maculata* seedlings caused by E11 (NSW2 in the present study). Most cankers were limited in extent and had healed completely three months after inoculation and it was concluded that *E. gyrosa* symptom development and severity depended on stress. However isolates used by Old *et al* (1990) E11, E18 and E9 (NSW2, TAS11 and VIC2 in the present study) behaved more aggressively with 12 month old *E. nitens* and *E. globulus* which were clearly healthy and unstressed. Mean lesion length at two months was

22.6 mm, 14.4 mm and 17.8 mm respectively on *E. nitens* seedlings. Although patterns in lesion lengths and tangential spread produced by the tested isolates on *E. nitens* seedlings were similar to those on *E. globulus*, the latter species appeared more susceptible to inoculation of *E. gyrosa* and other fungal species (Chapter 2).

- provenance and expressed virulence

Susceptibility variation in eucalypt provenances to other fungal pathogens has been detected. Jayasree *et al.* (1984) assessed 38 provenances of 15 eucalypt species to *Cylindrocladium* blight. Blum *et al* (1992) screened 41 provenances of 17 eucalypt species in growth chambers for resistance to damping-off by *Cylindrocladium clavatum* or *C. scoparium*. Golfari (1975) reported significant variation in resistance to *Cryphonectria cubensis* (as *Diaporthe cubensis*) among *Eucalyptus* species and provenances in plantations. Florence *et al.* (1986) found that the severity of infection by *C. cubensis* varied with eucalypt species and recommended planting resistant provenances. Virulence of *C. cubensis* was tested on 18 month old seedlings of *E. pellita* and two provenances of *E. saligna* by Alfenas *et al* (1983). They concluded that variation in resistance of *Eucalyptus* spp. was quantitatively isolate-specific.

Both *E. nitens* and *E. globulus* seedlings were of mixed provenance and variations in lesion size between individuals inoculated with the same isolate were often observed in the test. Significant variation in canker size caused by *E. gyrosa* isolates was found among *E. nitens* provenances on trees in the field inoculations (Chapter 2) and on 12-month-old seedlings in a shade house (Yuan unpublished data).

- management practices and expressed virulence

In the Tewkesbery plantation of origin, the *E. gyrosa* cankers had been unexpectedly numerous and severe when assessed in 1994. Previous to the canker epidemic the trees had been clearly noted as vigorous and healthy (Wardlaw 1998).

Although the outbreak of *E. gyrosa* canker disease at Tewkesbury may be a consequence of more virulent strains (isolates from these demonstrated a slightly greater comparative pathogenicity to others in artificial inoculations) it is also highly

probable that silvicultural practices, such as thinning and pruning played a significant role in the onset of the epidemic. Wardlaw (1998) found no differences at the Tewkesbery plantation in the level of canker infection between unpruned, unthinned plots and thinned, pruned plots. However he observed the epidemic at a late stage when already severe. It might have been the thinning in 1993 that created conditions (eg. wounds, pruning stress) leading to an initial inoculum build up. In addition, in 1993 the summer was very dry. Another predisposing factor was the planting of a provenance selection with rough bark which could provide ideal conditions for the perpetuation of inoculum within the bark layers and infection via bark cracks under conditions promoting host stress.

INFECTION ABILITY OF CONIDIA AND ASCOSPORES

This preliminary inoculation study demonstrated that wound inoculated ascospores and conidia can give rise to similar sized cankers. This indicates that they can act as infective propagules for dissemination of the pathogen in natural conditions. There did not appear to be any self-inhibition of spores at the higher concentration tested.

Bright-orange conidiomata of *E. gyrosa* are usually abundantly produced on the surface of induced cankers in inoculation tests, although perithecia have never been found on artificial cankers (Old *et al.* 1986; Yuan 1989; van der Westhuizen *et al.* 1993). Lesions resulting from both conidial and mycelial inoculation produced typical condiomata. The fact that there was no sporulation on any lesions of seedlings inoculated with ascospores in this test is somewhat unusual compared with conidial and mycelial inoculations.

Reisolations from the lesions induced by either ascospores and conidia produced conidiomata after two weeks in culture suggesting that mycelium developed from ascospores in wood tissue can produce conidiomata and conidia. It may have been possible to observe sporulation on surface of the lesions produced by ascospores if the length of the experiment had been extended. However the absence of sporulation and

comparatively higher occlusion rate in lesions resulting from ascospore inoculation may indicate that lesions were slower to establish. In many ascomycetes the ascospore is predominantly for survival and colonisation of new sites and spores may have a dormant period. In contrast conidia are most often dispersal spores with only a moderate capacity for dormant survival and germinate readily in the presence of nutrients (Carlile and Watkinson 1994). The fact that single ascospore *E. gyrosa* isolates were obtained by the author without recourse to pre-treatment (eg. heat as for *Neurospora* ascospores) may mean that ascospore dormancy cannot explain the observed differences in lesion development between spore types.

Since mycelium is not a natural inoculum, it could be argued that conidia or ascospores should be used as inoculum for pathogenicity tests for canker fungi. However, as demonstrated in this study, lesions were far more extensive with inoculated mycelium than with spore suspensions, mycelial inoculation is probably a useful practice for pathogenicity and resistance studies by the artificial inoculation of *E. gyrosa*. The benefit of using mycelium in screening is that standard conditions are critical for consistent disease expression allowing comparison to be made between isolates and host species. This is easier to achieve with mycelial inoculum which in the majority of cases can be easily produced in the required quantities in the laboratory. Ascospores of *E. gyrosa* cannot be obtained *in vitro*. Pronczuk and Messyasz (1991) found that spore inoculum of *Microdochium nivale* (Fr.) Samuels & Hallett was unable to cause disease in *Lolium perenne* L. while mycelial inoculum incited severe disease. They also concluded that spore inoculum requires longer incubation than mycelial inoculum and the latter is more useful for screening plants for resistance.

This study provides the technique and basis for the further investigation of the role and importance of conidia and ascospores in the disease cycle of *E. gyrosa*. Research has shown that fungal ability to sporulate on the surface of lesions, the number of spores produced and variations in the period spores are latent in plant tissue are important factors that condition both pathogenicity and epidemic development (Johnson and Taylor 1976; Rotem 1978; Zadoks and Schein 1979).

COLONY MORPHOLOGY, FUNGICIDE RESPONSE AND VEGETATIVE COMPATIBILITY GROUPS

Although fungicide resistance has been used as one of the characters to detect variation in species of other plant pathogenic fungi (Adaskaveg and Hartin 1997) or in populations of mycoparasitic fungi (Bonnen and Hopkins 1997), fungicide tests using cholorothalonil in the present study did not distinguish the isolates of *E. gyrosa* from different geographical origins or the morphological groups. However, the observed variations among the tested isolates in resistance to cholorothalonil indicates that this method could be still useful for the same purpose if other fungicides were applied at different concentrations.

Old and Dudzinski (unpublished data, 1991) noticed that based on colony colour, two types of *E. gyrosa* were isolated from wounded eucalypts in wounding trials at Orbost, Victoria, eg. orange-coloured and grey-colored isolates. In the present study, four types of colony morphologies based on both colour (orange/grey) and density (sparse/dense) were determined among 133 isolates from Australia and overseas. Generally, colony colour fell into the two colour types described by Old and Dudzinski. Some isolates with darker pigmented reddish brown colonies were however included with the orange-colored isolates.

Endothia gyrosa colony types appeared to have some association with geographical origins. The majority of the Tasmanian isolates fell into a single type, Type I (grey and dense mycelium). Most isolates from mainland Australia were in Type II (orange and sparse mycelium). No isolates from Australia fell into Type III (orange and dense mycelium), a group with only isolates from the USA and South Africa. Type IV (grey and sparse mycelium) only contained isolates from Australia: Tasmania, southeastern Australia and Western Australia.

Colony characteristics may not be very useful to distinguish meaningful groups among isolates of *E. gyrosa*. Cultural features of individual fungal isolates are known to differ in their degree of instability. Not all the cultural variation in fungi has a

nuclear basis (Caten 1996). However in *E. gyrosa* there did appear to be two distinct types of culture, one with orange pigmentation and another with a grey colour.

Nine VC groups were distinguished among the 16 *E. gyrosa* isolates tested on both 2% malt extract agar and PDAvc medium in the present study. Isolates originating from very distant geographic locations (different countries) were incompatible with each other.

A total of five VC groups were found among 11 Australian isolates, with isolates as geographically distant as Victoria and Western Australia in the same VC group (eg. TAS2, TAS3, TAS4, TAS5 and NSW in one group; VIC1, WA1 and WA2 in another group). This may be an indication of a low number of groups Australia wide. Fiely *et al.* (1995) identified 21 isolates of *Fusarium oxysporum* from geographically distant regions as Japan, Sweden and the United States into a single VC group. However Kubone (1991, unpublished data) in his investigation of *E. gyrosa* isolates from naturally infected trees in East Gippsland, Victoria, detected many VC groups.

In Kubono's work *E. gyrosa* isolates from the same tree were vegetatively compatible when paired within the same morphological groups but incompatible when paired with different morphological groups. He also reported reaction zone formation between all isolates from different trees even if they were from the same morphological group. VC reactions presenting between colonies of two different morphological groups as well as between colonies of the same morphological groups have been recorded for other fungal species (Brayford 1990). In this study there was overlap, although not consistent, of colony type and VC group among the 16 isolates. The compatible isolates from Victoria and Western Australia, for example, were grouped together morphologically.

Differences between the results obtained by Kubono may perhaps be explained by the use of different cultural techniques and interpretation to the present study. The nature of the zone line between two isolates observed by Kubono (1991, unpublished data) and scored as incompatible is not clear. Kubono used PDA as growth medium for *E.*

gyrosa VC group testing. Both MEA and PDA have been used for determining VC groups for many other fungal species of Ascomycotina and Basidiomycotina (Anagnostakis 1977; Rodrigures *et al.* 1995; Rizzo *et al.* 1995). However in the initial VC testing in this study, a problem was encountered (on both 2% MEA and PDA). Incompatible reactions, if present, could not be clearly visualised. Pairs of *E. gyrosa* isolates did not show clearly typical incompatibility reactions such as a barrage line bordered or not by fruiting bodies, a clear zone with or without a pigmented line between the pairing isolates (Anagnostakis 1977; Hopkin *et al.* 1989; Leslie 1993). On the PDAvc medium containing a pH indicator, a green line between incompatible pairing isolates due to change of pH caused by hyphal cell death (Powell 1995) made it possible to distinguish between VC groups of *E. gyrosa*.

Techniques for investigating VC groups for *E. gyrosa* could still be improved. Smit *et al.* (1997) used freshly prepared oatmeal agar to examine VC groups of *Diaporthe ambigua*. This medium was shown to display the best vegetative incompatibility reactions among five tested media including PDA, Water agar, Difco oatmeal agar, freshly prepared oatmeal agar (Smit *et al.* 1997). VC studies in the future may require more efficient methods, like complementation of recessive auxotrophic marker (Leslie 1993). The use of freshly isolated single spore isolates from condia produced *in vitro* can also improve the clarity of vc reactions.

Pycnidia were often observed forming along reaction lines for most of the incompatible *E. gyrosa* pairings. They were only found on one partner of the paired colonies even if the cultures were incubated for more than 8 weeks. Anagnostakis (1977) reported that pycnidia formed in two lines on either side of the pairing isolates of *C. parasitica*. If one member of the pair was hypovirulent, pycnidia formed first along the barrage zone on the virulent strain and much later on the hypovirulent.

Hypovirulent strains have been found containing dsRNA or *Hypovirus*, a kind of virus of the family *Hypoviridae* (*sensu* Hillman *et al.* 1995), which can be transmitted to virulent strains by hyphal fusions of vegetatively compatible strains. The recipient virulent or normal strains are often modified significantly in their morphology (Chen

et al. 1996). For example, the *E. gyrosa* isolate (cited by Chen *et al.* as hypovirus-free) used in their study (ATCC 48192, as USA1 in the present study) showed a noticeable change in its colony pigmentation to grey (very sparse) when transfected with a wild-type *Hypovirus CHV1-713* described originally from the Italian isolates of *C. parasitica* (Chen *et al.* 1996).

In the present pairing tests, no colony morphological changes were observed either between or within VC groups of all the *E. gyrosa* isolates tested. This might suggest there were no hypovirulent strains among the isolates of *E. gyrosa* studied here. Pathogenicity tests of Australian isolates of *E. gyrosa* conducted in Chapter 3 have also indicated that there is all very little intraspecific variation among isolates which are all capable of producing cankers on inoculated stems of *Eucalyptus nitens* and *E. globulus*. Further research should established if *E. gyrosa* isolates contain any endogenous virus-like double-stranded RNA genetic elements.

Analysis of VCGs in populations of filamentous ascomycetes has been used to assess whether a pathogen has been recently introduced into an area or whether it has been there for an extended time (Glass and Kuldau 1992). However, a more detailed investigation of the entire collection of Australian isolates, including both VC testing and DNA analysis, will be required for a valid discussion of such questions. It would also be difficult to draw any conclusions without knowledge about sexual regulation in *E. gyrosa*. A population study will also be carried out at a single site in Tasmania with *E. gyrosa* cankers using single ascospore and single ascus strains.

DNA POLYMORPHISM

Preliminary results of RFLP analysis of the ITS region of rDNA indicated that all the tested *E. gyrosa* isolates from both Australia and overseas are most likely conspecific.

Chen *et al.* (1996) included a *E. gyrosa* isolate (USA1) in their study of chestnut blight fungus *Cryphonectria parasitica* (Murr.) Barr using nuclear ribosomal DNA

nucleotide sequence analysis. That study provides a preliminary data on ITS sequences with the 18S gene for *E. gyrosa*. A further investigation of *E. gyrosa* isolates using DNA sequencing methods is needed to investigate fragment size polymorphisms and restriction sites.

If RFLP patterns of the isolates are compared, one South African isolate (SA1) was similar to the Australian isolates and the other (SA2) closer to the American isolates. This agrees with classification by isolate colony morphology. SA1 has the same colony type (type I) as those from Tasmania while SA2 has colony type as type III resembling the American isolates.

In RAPD analysis, isolates from South Africa were closer to the Australian isolates (BSC = 48.2%-66.5%) than isolates from USA and Italy (BSC = 41.5%-51.8%).

All the above observations suggest that the *E. gyrosa* isolates investigated in the study were derived from a relatively recent common ancestor. The Australian *E. gyrosa* may be a recent introduction from North America via South Africa. Or an indigenous Australian *E. gyrosa* may have been introduced with eucalypts to South Africa. A comprehensive RAPD analysis of more isolates from different geographical locations would be necessary to confirm this hypothesis.

Genetic variation was observed among the Australian isolates. For example, six Tasmanian isolates fell into two discrete clusters in RAPD analysis, while two Western Australian and one Victorian isolates were closely clustered into one group. Similar variation within *E. gyrosa* isolates has been observed in a previous study by Davison and Coates (1991) using isozyme analysis. Six *E. gyrosa* isolates examined were divided into three clusters. One isolate from Tasmania was allozymically identical to two isolates from Western Australia and another from New South Wales in one cluster. The other two isolates, one from Australian Capital Territory and one from New South Wales fell separately into two discrete clusters.

RAPD groups did not appear to be associated with colony morphology. However, there was an overlap between vegetative compatibility and RAPD groups for Australian isolates. For example, the four Tasmanian isolates (TAS2-5) were always clustered in one group in both VC and RAPD analyses. Two Western Australian isolates (WA1, WA2) and the Victoria isolate (VIC1) formed one VC or RAPD group. These three isolates also fell into one colony type. Leslie (1993) states that if strains within a VC group are all closely related or identical by other measures of genetic variability, eg. RFLPs or RAPDs, then these strains are all derived from a common progenitor.

RAPD markers have been used to differentiate other fungal races into groups directly related to virulence (Assigbetse *et al.* 1994). In the present study, the Australian isolates were divided into two discrete clusters The first cluster included six isolates, most of which were pathogenic, except for ACT1, a less aggressive isolate (see Chapter 2) and TAS6, the pathogenicity of which was not determined. In contrast, in the second cluster the three isolates, TAS3, TAS4 and NSW1 were all less pathogenic. It may be possible to use RAPD markers to differentiate isolates of *E. gyrosa* according to their pathogenicity. Wronski *et al.* (1997) examined 34 Austrian isolates of *Cryphonectria parasitica*, a closely related species to *E. gyrosa* (Chen *et al.* 1996) by RAPD technique and other conventional methods. They proved that RAPD analysis is an efficient method for distingushing between different *C. parasitica* genotypes, although no direct correlations between the RAPD markers and virulence were found for *C. parasitica.*

POTENTIAL THREAT OF *E. GYROSA* TO *E. GLOBULUS* AND *E. NITENS* PLANTATIONS

Surveys during this study in Chapter 1 showed that *E. gyrosa* was frequently isolated from both diseased and healthy tissue of *Eucalyptus* spp. in Tasmania. All *E. gyrosa* isolates including less pathogenic isolates, such as VIC2, NSW1 and ACT1 were readily recovered from tissue (both necrotic bark and discoloured xylem) around lesions, and persisted in lesions for at least 7 months. These results indicate that

potential inoculum sources are widely distributed throughout the island. If conditions become favourable, current knowledge points to *E. gyrosa* as being an extremely successful opportunist.

There does not appear to be a high level of intraspecific variation and differences in the level of pathogenicity appear to be more a function of host response. However with the sexual reproduction in southeast Australia there is a possibility of genetic variation and the natural selection of more virulent strains.

The results of pathogenicity tests in Chapter 2 and in this study indicate that both *E. nitens* and *E. globulus* are susceptible to *E. gyrosa*. As indicated in Chapter 2, *E. nitens* and *E. globulus* plantations for sawlog and veneer are increasing rapidly in Tasmania and Western Australia (Burns *et al.* 1997). Such plantations (which must be pruned and thinned) are very economically sensitive to stem defects caused by cankers and associated problems such as stem decay. Most of these plantations are still first rotation crops and cankers have not yet been signalled as a problem. However if *E. gyrosa* inoculum builds-up into subsequent rotation crops there is a real possibility of increasing levels of damage.

CHAPTER 4: RESEARCH OUTCOMES

This survey has shown that a wide range of fungal species were associated with stem cankers of *Eucalyptus* spp. in forests and plantations in Tasmania. Several of the fungi obtained in this survey are known to be pathogenic or belong to genera containing recognised pathogens, eg. *Aulographina eucalypti*, *Endothia gyrosa*, *Harknessia* cf. *eucalypti*, *Phoma* sp., *Seiridium eucalypti*, and *Zythiostroma* sp.

The majority of the fungal species tested in the present study are weak pathogens or saprophytes. They did not produce severe canker lesions on either seedlings or trees of eucalypts. These fungi are persistent in both living and dead tissue around inoculation wounds (even after the lesions had occluded).

Variability in disease resistance was found among *E. nitens* provenances, although the impacts of infections were small. *E. nitens* appears more resistant to infection with canker fungi than *E. globulus*.

Smooth-barked trees of *E. nitens* were more susceptible to post-penetration development of canker fungi than were rough-barked trees. The larger external lesions on smooth-barked trees found during this study is apparently due to differences in bark anatomy between rough- and smooth-barked trees. Longitudinal cracks on bark surfaces of rough-barked trees may provide suitable infection courts as natural "wounds" for *E. gyrosa* which is a wound fungus.

Interactions between factors such as site, host species, provenance and bark type influence formation of cankers in stems of inoculated trees, as demonstrated in the present study. The limited penetration of these fungal infections to bark tissue on large, healthy trees indicates that the fungal species tested in the present study are probably opportunists. Healthy, vigorous trees are unlikely to be markedly affected by these opportunists, unless stressful events, such as drought and insect defoliation occur. It is unlikely therefore that breeding programs will consider canker resistance

as a priority although if tree improvement follows the clonal track as in South Africa and many parts of the world, then some selection of clones on the basis of susceptibility to these fungi may be needed.

All *E. gyrosa* isolates are capable of causing lesions on artificially inoculated eucalypt seedlings. Their pathogenicity does not appear to be linked to geographic origin or host origin. Three isolates, TAS1 from Tasmania, WA1 from Western Australia and VIC1 from Victoria are more pathogenic than others in artificial inoculation tests, although intraspecific variation in pathogenicity is low. *Eucalyptus globulus* and *E. nitens* are susceptible to *E. gyrosa*.

The conidia and ascospores of *E. gyrosa* have proven to be able to infect *E. nitens* seedlings when applied to wounds, indicating that both spore forms are infective and dispersive propagules under natural conditions. The technique developed for inoculating ascospores and conidia can be employed in future infection studies. However artificial inoculation with mycelium is probably a more useful practice for pathogenicity and resistance studies with *E. gyrosa*.

Fungicide response assays using cholorothalonil did not show differences between the isolates from different origins. The morphology of vegetative mycelium divided 133 *E. gyrosa* isolates from overseas and Australia into 4 groups based on colony colour (grey or orange) and density.

A technique for detecting VC groups in *E. gyrosa* was developed. A low number of VC groups were detected among the Australian isolates paired. Isolates from geographically distant locations in Australia, such as Western Australia and Victoria were vegetatively compatible. In general, a sexual reproducing population would be expected to have a high level of VC group diversity (Leslie 1993).

RFLP analysis of the PCR amplified ITS1-ITS4 region of rDNA indicates that *E. gyrosa* isolates from different origins in the USA, Europe, South Africa and

Australia are conspecific despite differences in colony morphology, vegetative compatibility and host specificity. There were slight differences in the size of amplified fragments which must be further investigated with DNA sequencing.

RAPD analysis also revealed a close relationship between Australian and overseas isolates, especially with a South African isolate. Another South African isolate was grouped closer to a North American isolate. *E. gyrosa* in Australia could have been introduced from North America via South Africa. There was some overlap with differentiation by investigating DNA polymorphisms and VC and vegetative morphology groups determined.

The majority of large scale *E. nitens* or *E. globulus* plantations in Tasmania, whether intended for sawlog or pulp, have been recently planted and most trees are aged under 15 years. Currently there are no reports of serious disease problems caused by canker fungi in Tasmanian plantations but certain of the canker fungi found in the survey may constitute a threat to an expanding plantation forest estate. *E. gyrosa,* an excellent opportunist and reported in South Africa as a serious canker pathogen, is considered as the canker fungus with the highest potential for damage in Tasmania (and mainland Australia). Recent reports of damage to healthy young *E. globulus* plantations from Western Australia support this view.

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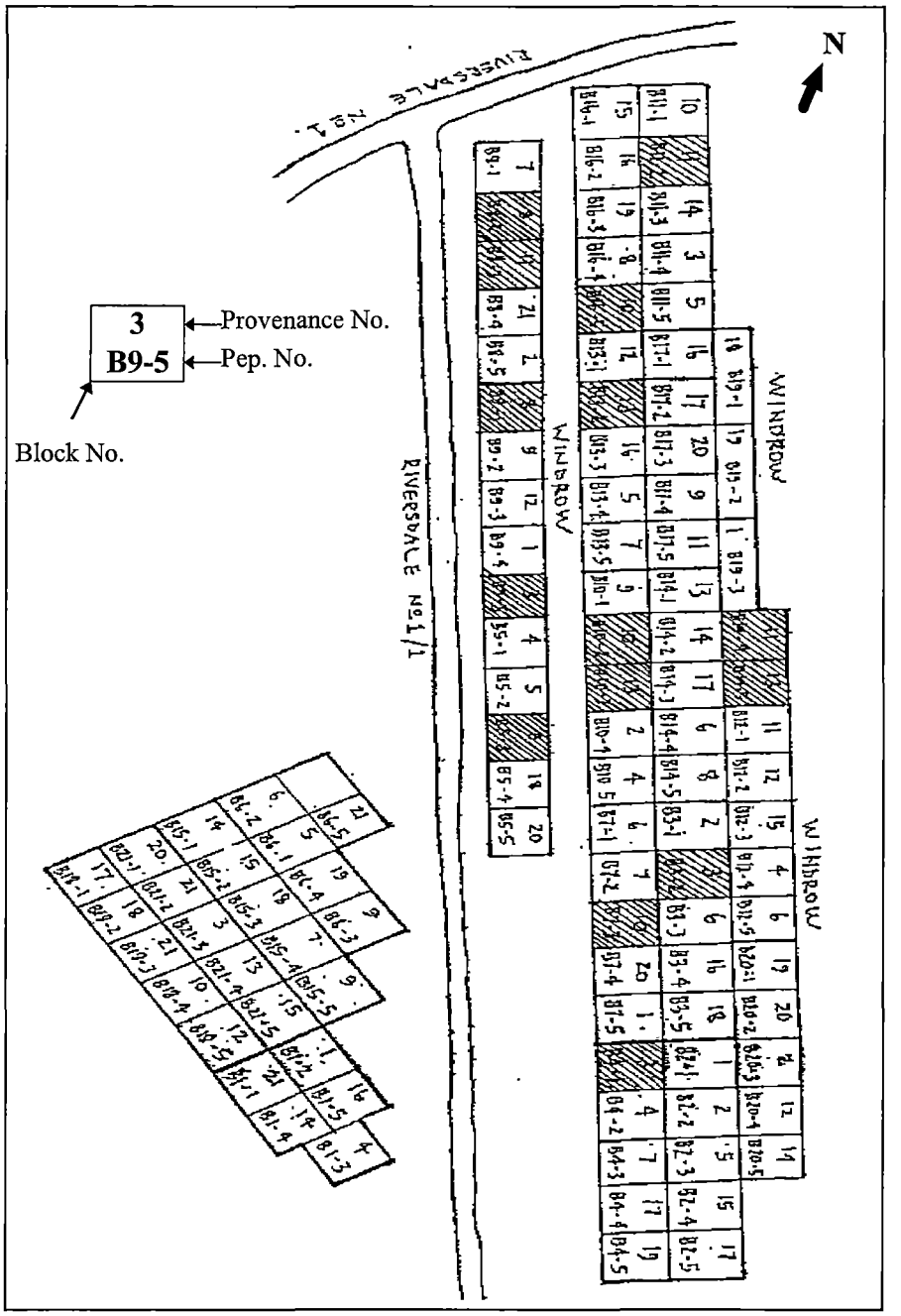
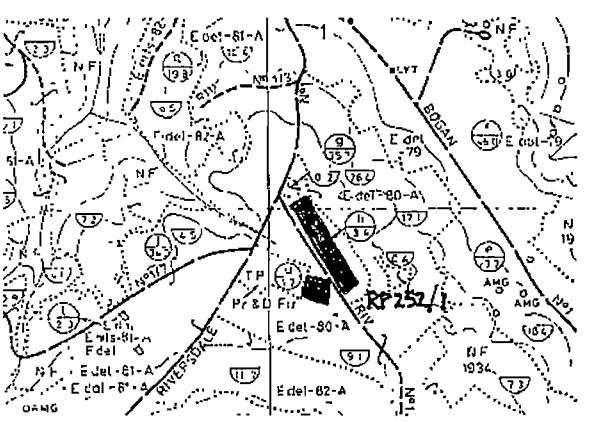
Appendix 1: *Eucalytus nitens* provenance trial (RP 252-1) at Liffey, Deloraine District used for inoculation tests of canker fungal species.

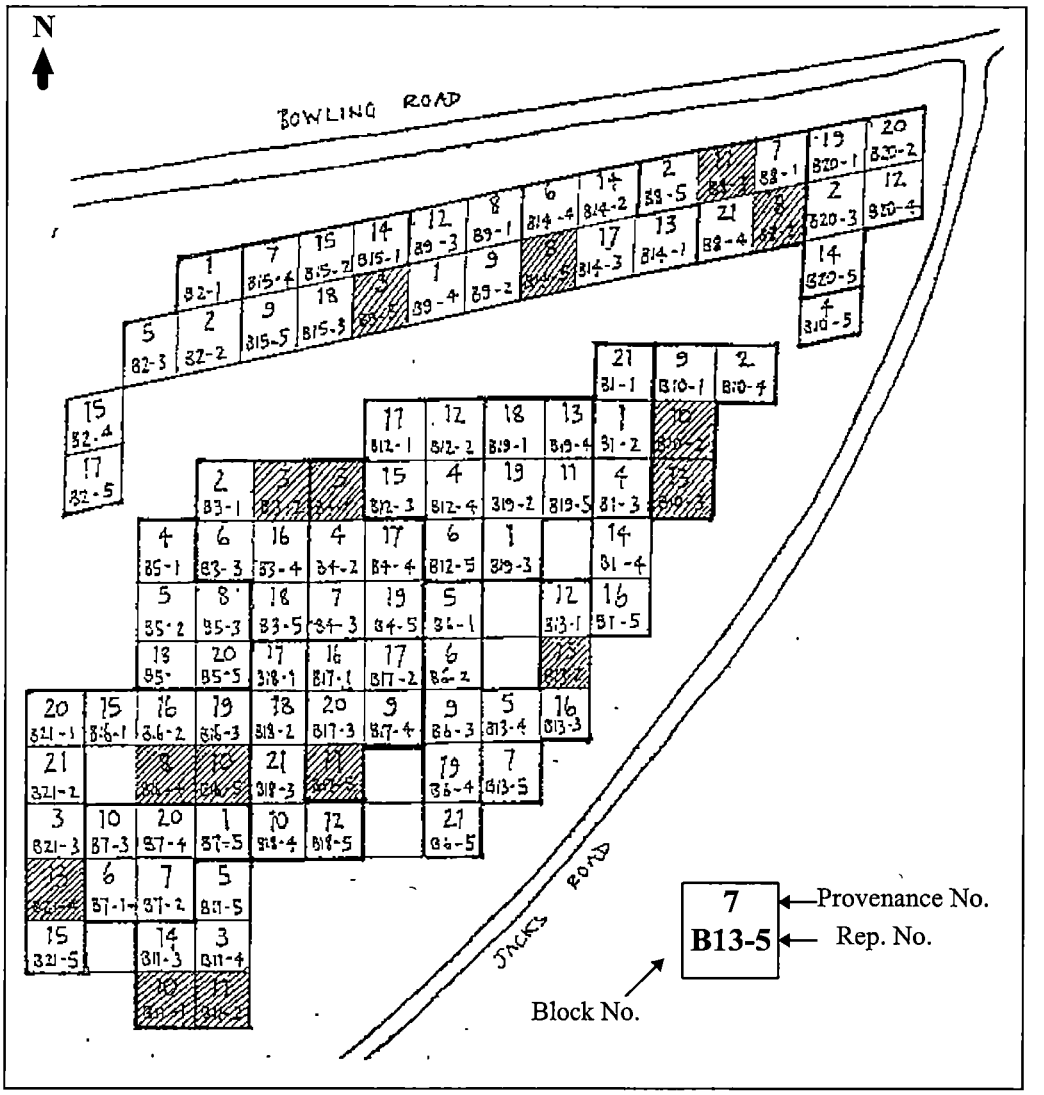


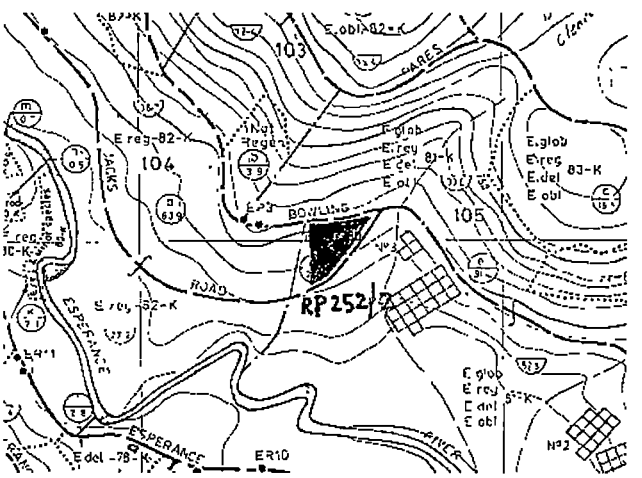


The trial is located at north Tasmania near Deloraine and was established in 1980. Plot size is 15 m x 18 m with 5 x 6 trees at 3 x 3 m spacing. Plots were replicated 5 times and laid out as an incomplete Latin square design.

Trees in the shadowed plots, No.3, No.8, No.10, No.11 and No.13 were selected for inoculating.

Appendix 2: ***Eucalyptus nitens*** **provenance trial (RP 252-2) at Esperance, Geeveston District used for inoculation tests of canker fungal species.**





The trial is located at south Tasmania near Dove (see the map) and was established in 1980. Plot size is 15 x 15 m with 5 x 5 trees at 3 by 3 m spacing. Plots were replicated 5 times and laid out as an incomplete Latin square design.

Trees in the shadowed plots, No.3, No.8, No.10, No.11 and No.13 were selected for inoculating. Three plots each provenance were used.

Appendix 3: Analyses of variance for inoculation tests of stem fungi

Table 1: Analysis of variance of lesion length, tangential spread and internal discolouration length for pathogenicity tests of 13 fungal species (17 isolates) inoculated into 12-month-old *Eucalyptus nitens* seedlings (NE-1) assessed at two times

		Lesion length	Tangential spread		Length of internal discolouration
Source of variation	df	MS	MS	df	MS
Isolate	17	1763.27**	62775**	17	1143.8**
Time	1	1.61	17		
Replicate (Block)	4	51.71	463	4	224.3
Isolate x Time	17	218.12**	3450**		
Error	140	88.07	1310	68	84.1

** = Significance at the 1% level.

Table 2: Analysis of variance of lesion length, tangential spread and internal discolouration length for pathogenicity tests of 13 fungal species (17 isolates) inoculated into 12-month-old *Eucalyptus globulus* seedlings assessed at seven months

		Lesion length	Tangential spread		Length of internal discolouration
Source of variation	df	MS	MS	df	MS
Isolate	17	971.05**	6694.1**	17	1143.8**
Replicate (Block)	4	19.16	641.5	4	224.3
Error	68	26.7	318.5	68	84.1

** = Significance at the 1% level.

Table 3: Analysis of variance of lesion length for pathogenicity tests of 13 fungal species (17 isolates) inoculated into 12-month-old seedlings of *Eucalyptus nitens* and *E. globulus* assessed at seven months

Source of variation	df	MS	F	$F_{0\,05}$	$F_{0\,01}$
Eucalypt species	1	236.9	3.92*	3.84	6.64
Isolates	17	1321.0	21.86**	1.57	1.87
Replicate (Block)	4	73.7	1.22	2.37	3.32
Species x isolates	17	243.4	4.03**	1.57	1.87
Error	140	60.4			

* = Significance at the 5% level; ** = Significance at the 1% level.

Table 4: Analysis of variance of lesion area (cm^2) for pathogenicity test of 9 fungal species (11 isolates) inoculated into 5 provenances of 16-year-old *Eucalyptus nitens* at two locations, assessed at two different times.

Source of variation	df	MS	F	$F_{0\,05}$	$F_{0\,01}$
Time	1	1564.4	125.2**	3.84	6.64
Site	1	1231.8	98.5**	3.84	6.64
Bark	1	1549	123.7**	3.84	6.64
Provenance	4	39.9	3.1*	2.37	3.32
Fungus	11	2713.5	217.1**	1.79	2.24
Replicate (Block)	2	4.11	0.3	2.99	4.60
Time x fungus	11	190.3	15.2**	1.79	2.24
Site x fungi	11	406.6	32.5**	1.79	2.24
Bark x fungi	11	184.2	14.7**	1.79	2.24
Porv. x fungi	44	20.2	1.6**	1.35	1.52
Error	1342	12.5			

* = Significance at the 5% level; ** = Significance at the 1% level.

Table 5: Analysis of variance of lesion area (cm^2) on smooth- and rough-barked stems of three 16-year-old *Eucalyptus nitens* provenances inoculated with conidia and mycelium of three *Endothia gyrosa* isolates on intact and superficially wounded bark surface assessed six months after inoculation

Source of variation	df	MS	F	$F_{0\,05}$	$F_{0\,01}$
Inoculation type	1	2169.3	93.9**	3.84	6.64
Provenance	2	37.7	1.6	2.99	4.60
Isolate	3	858.4	37.2**	3.84	6.64
Bark	1	2110.4	91.6**	3.84	6.64
Replicate (Block)	2	58.9	2.6	2.99	4.60
Inoculation type x Isolate	3	394.3	17.1**	2.60	3.78
Provenace x Isolate	6	6.2	0.3	2.09	2.80
Isolate x Bark	3	329.5	14.3**	2.60	3.78
Inoculation type x Bark	1	954.3	41.4**	3.84	6.64
Error	121	23.1			

** = Significance at the 1% level.

Appendix 4: List of *Eucalyptus* species on which *Endothia gyrosa* or *Endothiella gyrosa* naturally occur in Australia

Species	ACT	NSW	TAS	VIC	WA	References
E. blakelyi	+	+				Old *et al* 1986; Yuan 1989
E. bridgesiana		+				Yuan 1989
E. delegatensis		+	+			Old *et al* 1986;
E. calophylla					+	Davison & Tay 1983
E. fastigata		+				Yuan 1989
E. globoidea				+		Old *et al* 1993
E. gomphocephala					+	Fraser & Davison 1986
E. maculata		+				Old *et al* 1986;
					+	Fraser & Davison 1986
E. marginata					+	Davison & Tay 1983
E. obliqua			+			Old *et al* 1986;
E. pauciflora		+				Old *et al* 1986
E. regnans			+	+		Old *et al* 1986; Yuan 1989;
E. rossii		+				Old *et al* 1986;
		+				Yuan 1989
E. saligna		+				Old *et al* 1986;
					+	Fraser & Davison 1986
E. sieberi				+		Old *et al* 1986, 1993; Yuan 1989
E. stellulata		+				Yuan 1989
E. viminalis		+				Old *et al* 1986;
E. wandoo					+	Fraser & Davison 1986

Appendix 5: Details of *Endothia gyrosa* isolates investigated in colony morphology study

Isolate No .	Host	Collection location
Australian isolates		
E10	*E. pauciflora*	Corin Rd, ACT
E12	*E. pauciflora*	Brindabella, ACT
E13	*E. rossii*	Ingledene, ACT
E14	*E. viminalis*	Brindabell, ACT
E29; CF65	*E. blakelyi*	Uriarra Rd, ACT
CF59	*E. bridgesiana*	Walcha, NSW
CF60	*E. stellulata*	Walcha, NSW
E4	*E. saligna*	Termeil, NSW
E8; E25	*E. delegatensis*	Batlow, NSW
E11	*E. saligna*	Currowan SF, NSW
ECF1-ECF28	*E. nitens*	St. George's Rd, TAS
ECF61-ECF65	*E. regnans*	Westfield, TAS
ECF66-ECF70	*E. nitens*	Wages Rd, TAS
ECF156-ECF196	*E. nitens*	St. George's Rd, TAS
ECF197-ECF215	*E. nitens*	Camden, TAS
ECF232, ECF233	*E. nitens*	St. George's Rd, TAS
ECF234	*E. regnans*	Westfield, TAS
ECF254	*E. nitens*	Basil's Rd, TAS
E18	*E. regnans*	Lone Star, TAS
E9	*E. viminalis*	Wombat SF, VIC
CF22	*E. sieberi*	Pheasant Tk, VIC
Orbost 15a; Orbost406	*E. sieberi*	Towser Ck Rd, VIC
Orbost71	*E. sieberi*	Fall's Ck, VIC
Orbost322; Orbost549	*E. globoidea*	Fred's Tk, VIC
WA2	*E. gomphocephala*	?, WA
WA20	*E. maculata*	?, WA
WA29	*E. saligna*	?, WA
WA54, WA55	*E. calophylla*	?, WA
WA25, WA60	*E. wandoo*	?, WA
Non Australian isolates		
CBS 250.54	*Castanea crenata* var. *tamba*	Corgomom, Italy
CBS 251.54	*C. crenata* var. *tamba*	La Coruna, Italy
ATCC48192	*Quercus palustris*	Virginia, USA
CBS 508.76 (ATCC48194)	*Quercus palustris*	Virginia, USA
CBS 509.76	*Quercus palustris*	Virginia, USA
CRY57;CRY62; CRY94	*E. grandis*	Sabie, SA
CRY103	?	Kwambonambi, SA
CRY232; CRY286;	?	Bloemfontein, SA
CRY287	?	Sabie, SA

All **ECF** isolates were collected from Tasmania; the isolates with **CF**, **E**, **Orbost**, **WA** designation are from other states of Australia and deposed at CSIRO, Forestry & Forest Products, Canberra, ACT; **ATCC** = American Type Culture Collection at Maryland, USA; **CBS** = Centraalbureau voor Schimmelculturees at Baarn, Netherlands; **CRY** isolates are from University of the Orange Free State, South Africa.

Appendix 6: Analyses of variance for inoculation tests of *Endothia gyrosa* isolates

Table 1: Analysis of variance of lesion length, tangential spread and internal discolouration length for pathogenicity test of 16 *Endothia gyrosa* isolates inoculated into 12-month-old *Eucalyptus nitens* seedlings assessed at two different times

		Lesion length	Tangential spread		Length of internal discolouration
Source of variation	df	MS	MS	df	MS
Time	1	118.61	871		
Isolate	16	681.87**	34267**	16	1087.3**
Replicate (Block)	4	48.05	3068	4	144.5
Isolate x Time	16	4.80	703		
Error	132	32.64	1854	64	86.1

** = Significance at the 1% level.

Table 2: Analysis of variance of lesion length, tangential spread and internal discolouration length for pathogenicity test of 16 *Endothia gyrosa* isolates inoculated into 12-month-old *Eucalyptus globulus* seedlings assessed at seven months

		Lesion length	Tangential spread		Length of internal discolouration
Source of variation	df	MS	MS	df	MS
Isolate	16	729.9**	6733**	16	1517.4**
Replicate (Block)	4	39.2	1352	4	66.9
Error	64	58.3	1066	64	122.2

** = Significance at the 1% level.

Table 3: Analysis of variance of lesion length for pathogenicity tests of 16 *Endothia gyrosa* isolates inoculated into 12-month-old seedlings of *Eucalyptus nitens* and *E. globulus* assessed at seven months

Source of variation	df	MS	F	$F_{0.05}$	$F_{0.01}$
Eucalypt species	1	1664.9	34.4**	3.84	6.64
Isolates	16	840.8	17.4**	1.64	1.99
Replicate (Block)	4	23.1	0.5	2.37	3.32
Species x isolates	16	247.9	5.1**	1.64	1.99
Error	132	48.5			

* = Significance at the 5% level; ** = Significance at the 1% level.

Table 4: Analysis of variance of lesion length (mm) on seedling stems of *Eucalyptus nitens* inoculated with conidia, ascospores and mycelium of three *Endothia gyrosa* isolates

	Ascospore and conidia		Mycelial inoculum	
Source of variation	df	MS	df	MS
Inoculation type	1	9.5		
Provenance	2	33.2**	2	215.6*
Isolate	3	188.9**	2	344.9**
Concentration	1	5.4		
Replicate (Block)	4	5.6	4	57.3
Prov. x Concentration	2	1.2	2	228.6*
Prov. x Isol.	6	9.6	4	71.9
Inoc. type x Isol.	3	4.5		
Isolate x Concentration	3	1.3		
Error	214	5.1	74	62.9

* = Significance at the 5% level; ** = Significance at the 1% level.